



# BEST AVAILABLE COPY

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

UMAÑA *et al.*

Appl. No.: 10/633,697

Filed: August 5, 2003

For: **Glycosylation Engineering of  
Antibodies for Improving Antibody-  
Dependent Cellular Cytotoxicity**

Confirmation No.: 5455

Art Unit: 1636

Examiner: Guzo, D.

Atty. Docket: 1975.0010005/TJS

## Declaration Under 37 C.F.R. § 1.132 of Pablo Umaña, Ph.D.

Commissioner for Patents  
PO Box 1450  
Alexandria, VA 22313-1450

Sir:

I, Pablo Umaña, Ph.D., declare and state as follows:

1. I am an inventor of the above-captioned application, U.S. Patent Application Number 10/633,697, filed August 5, 2003, entitled "Glycosylation Engineering of Antibodies for Improving Antibody-Dependent Cellular Cytotoxicity."
2. I am currently Chief Scientific Officer and Member of the Board at GlycArt Biotechnology AG in Zurich, Switzerland. I am an expert in the fields of molecular biology and immunology, with particular expertise in the area of antibody engineering. My qualifications as an expert are established by the attached *curriculum vitae* and the publications listed therein. (Exhibit A)
3. I have read and understood the subject matter of U.S. Pat. Appl. No. 10/633,697.
4. Glycoengineered antibodies produced according to the techniques we described in the above-captioned patent application are characterized by a substantial increase in the proportion of nonfucosylated oligosaccharides in their Fc region and

exhibit significantly increased binding to Fc receptors and antibody-dependent cellular cytotoxicity. Our studies confirming this are set forth in the attached Exhibits B-D.

5. The present application teaches methods for producing recombinant antibodies with increased Fc-mediated cellular cytotoxicity and/or increased Fc receptor binding by engineering a host cell to express an antibody and to have an altered level of activity of at least one glycoprotein-modifying glycosyltransferase. According to one embodiment, the application teaches that the altered level of glycosyltransferase activity is achieved by expression of at least one glycoprotein-modifying glycosyltransferase. The application specifically identifies GnT III and ManII as useful glycoprotein modifying glycosyltransferases for this purpose. In addition, the application teaches that glycoengineered antibodies can be obtained by coexpressing the antibody with *multiple* glycoprotein-modifying glycosyltransferases, such as GnTIII + ManII. (See page 13, lines 18-30; page 21, lines 15-25.)

6. Exhibit B is U.S. Pat. Appl. Pub. No. U.S. 2004/0241817, which published December 2, 2004, and is entitled "Fusion Constructs and Use of Same to Produce Antibodies with Increased Fc Receptor Binding Affinity and Effector Function." I am a named inventor on that application. Example 6 of the application describes experiments that I and my colleagues conducted to study the effect of coexpression in a host cell of the human  $\alpha$ -Mannosidase II ("hManII") gene or, alternatively, a polynucleotide encoding the Golgi localization domain of hManII fused to the catalytic domain of human galactosyltransferase, and an expression vector encoding the recombinant anti-CD20 antibody C2B8.

7. Specifically, to study the effect of hManII coexpression, we transfected HEK 293-EBNA cells with expression vectors encoding each of the light chain and

heavy chain of the anti-CD20 monoclonal antibody and the hManII gene. At day 5 post-transfection, supernatant was harvested and the monoclonal antibody was purified from the supernatant by sequential chromatography. (Paragraph Nos. 0434 - 0436.)

8. We analyzed the oligosaccharide structures of the purified antibody by enzymatically cleaving the oligosaccharides from the antibodies by PNGaseF digestion and, optionally, EndoH glycosidase digestion. The enzymatic digests containing the released oligosaccharides were subsequently analyzed by MALDI/TOF-MS. (Paragraph Nos. 0437-0446.)

9. The oligosaccharide profile of the anti-CD20 antibody coexpressed with hManII and the relative percentages of the structures found associated to the Fc portion of the antibody are shown in FIG. 34. As noted in the application, the oligosaccharides found associated to the Fc portion of the antibody are complex structures, 48% of which lack core fucose. The  $\alpha$ -Mannosidase II competes with the core-fucosyltransferase, generating 48% non-fucosylated oligosaccharide structures. In the absence of  $\alpha$ -Mannosidase II, the oligosaccharides of the Fc portion of the antibody are composed of only fucosylated structures (wild-type antibody). (See Paragraph No. 0448).

10. The oligosaccharide profile of the anti-CD20 antibody coexpressed with the hManII-GalT fusion protein is shown in FIG. 35A-B. Antibodies produced in the presence of hManII-GalT show almost 100% non-fucosylated structures. (Paragraph No. 0449.)

11. We also assayed antibodies produced in the presence of hManII or hManII-GalT fusion protein for binding to Fc receptors and ADCC activity. As shown in FIG 29, expression of a ManII-GnTIII fusion polypeptide in the antibody-producing host cells resulted in an increase in ADCC activity. (Paragraph No. 0419.) The binding

assay experiment utilized the cell line CHO-1708-37, which expresses on its surface the Fc $\gamma$ RIIIA receptor (CD16) and the  $\gamma$  chain of the Fc $\gamma$ RI receptor. The expression of the Fc $\gamma$ RIIIA receptor was assessed by FACS analysis using the 3G8-FITC monoclonal antibody. As shown in FIG. 36, the anti-CD20 antibody produced in the presence of  $\alpha$ -Mannosidase II binds to the Fc $\gamma$ RIIIA receptor with higher affinity than the wild-type antibody that was not produced in the presence of ManII. (Paragraph No. 0457.)

12. Exhibit C is a paper by my colleagues and me that was published in the journal, *Biotechnology & Bioengineering* 93:851-61 (2006). The title of the paper is "Modulation of Therapeutic Antibody Effector Functions by Glycosylation Engineering." In general, it describes the generation of anti-CD20 antibody glycovariants by coexpression in a host cell of GnT-III or chimeric GnTIII proteins.

13. In these studies, we analyzed two anti-CD20 antibodies produced by either: 1) coexpression with a fusion protein comprising the catalytic domain of human GnTIII fused to the Golgi localization domain of human Mannosidase II (termed "Glyco-1" antibodies); or 2) coexpression with the GnT-III/ManII fusion construct and a gene encoding ManII ("Glyco-2" antibodies) for ADCC activity and for their affinity to bind Fc $\gamma$ RIIIa compared to either the unmodified (*i.e.*, not glycoengineered) antibody, or to an antibody glycovariant produced under the same conditions by co-expression of GnT-III ("Glyco-0"). (Pages 856-857.)

14. We evaluated ADCC of the glycoengineered antibodies and found that both the Glyco-1 and Glyco-2 antibodies mediate an enhanced ADCC response. (Page 857; FIG. 5A.) The Glyco-1 and Glyco-2 variants were also 100-fold more potent than unmodified antibodies in depleting B-cells. (Page 857; FIG 5C.)

15. We also evaluated the binding of the glycoengineered antibodies to Fc $\gamma$ RIIIA on peripheral human natural killer (NK) cells, which are known to be important mediators of ADCC, and to constitutively express Fc $\gamma$ RIIIA. Both Glyco-1 and Glyco-2 were found to bind with a considerably higher affinity to NK cells than unmodified antibody. (See FIG. 4A). The Glyco-0 antibody produced by wild-type GnT-III coexpression was found to have intermediate Fc $\gamma$ RIIIA binding affinity between that of the unmodified antibody and Glyco-1/Glyco-2. (Page 857.)

16. Exhibit D shows the results of a study we conducted to determine the effect of constitutive coexpression of recombinant, wild-type  $\beta$ 1,4-N-acetylglucosaminyltransferase III (GnT-III) and Golgi  $\alpha$ -mannosidase II (ManII) in stable, industrial grade CHO cells on a recombinant anti-CD20 antibody produced by those cells.

17. FIG. 1 of Exhibit D shows the glycosylation profile (as determined by MALDI/TOF-MS) for the Fc-region oligosaccharides of a nonglycoengineered recombinant antibody produced in CHO cells. The level of nonfucosylated oligosaccharides is below 10%, which is typical for a nonglycoengineered antibody produced by CHO cells.

18. FIG 2 of Exhibit D shows the glycosylation profile for the Fc-region oligosaccharides of a *glycoengineered* anti-CD20 antibody produced in CHO cells. The glycoengineered antibodies had significantly increased levels of nonfucosylated oligosaccharides relative to the nonglycoengineered antibodies. In particular, peaks at m/z 1339, 1543 and 1705 correspond to nonfucosylated, complex oligosaccharides, which represent over 70% of the total oligosaccharides.

19. FIG. 3 of Exhibit D depicts the binding to Fc $\gamma$ RIII receptors achieved by the glycoengineered antibodies compared to the nonglycoengineered antibodies. Substantially increased binding to Fc $\gamma$ RIII receptors was observed for the glycoengineered antibody.

20. FIG. 4 of Exhibit D depicts the increased antibody-dependent cellular cytotoxicity observed for a glycoengineered antibody with increased levels of nonfucosylated Fc-region oligosaccharides compared to the nonglycoengineered antibody.

21. Thus, the attached Exhibits show that anti-CD20 antibodies coexpressed either with wild-type hManII or wild-type GnT-III or wild-type GnTIII and wild-type ManII have substantially increased affinity for the Fc $\gamma$ RIIA receptor and enhanced ADCC activity compared to the corresponding nonglycoengineered antibody. This demonstrates that the techniques taught in the present application can be used to produce glycoengineered recombinant antibodies having increased Fc-mediated cellular cytotoxicity (e.g., ADCC activity) and increased binding affinity for Fc receptors.

22. The undersigned further declares that all statements made herein of his knowledge are true, and that all statements made on information and belief are believed to be true, and that these statements were made with the knowledge that willful false statements or the like so made are punishable by fine, imprisonment, or both under § 1001 of Title 18, United States Code, and that such willful false statements may jeopardize the validity of any patent application or patent issued thereon.

August 20, 2006  
Date

  
\_\_\_\_\_  
Pablo Umaña, Ph.D.



## Supplemental Declaration for Patent Application

Docket Number: 1975.0010005/TJS/T-M

As legal representative of named inventor, James E. Bailey, I hereby declare that:

My residence, mailing address, and citizenship are as stated below next to my name.

I believe James E. Bailey was an original, first and joint inventor of the subject matter that is claimed and for which a patent is sought on the invention entitled **Glycosylation Engineering of Antibodies for Improving Antibody-Dependent Cellular Cytotoxicity**, the specification of which is attached hereto unless the following box is checked:

was filed on August 5, 2003;  
as United States Application Number 10/633,697; and  
was amended on August 5, 2003 and November 1, 2005 (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information that is material to patentability as defined in 37 C.F.R. § 1.56, including for continuation-in-part applications, material information which became available between the filing date of the prior application and the national or PCT international filing date of the continuation-in-part application.

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or (f) or § 365(b) of any foreign application(s) for patent, inventor's or plant breeder's rights certificate(s), or § 365(a) of any PCT international application, which designated at least one country other than the United States of America, listed below, and have also identified below, by checking the box, any foreign application for patent, inventor's or plant breeder's rights certificate(s), or PCT international application having a filing date before that of the application on which priority is claimed.

### Prior Foreign Applications(s):

			Priority Claimed
(Application No.)	(Country)	(Day/Month/Year Filed)	<input type="checkbox"/> Yes <input type="checkbox"/> No
			<input type="checkbox"/> Yes <input type="checkbox"/> No
(Application No.)	(Country)	(Day/Month/Year Filed)	

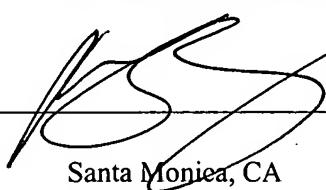
Send Correspondence to:                    Customer No. 26111  
STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.  
1100 New York Avenue, N.W.  
Washington, D.C. 20005-3934

Direct Telephone Calls to: (202) 371-2600

I hereby declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of first Inventor:	Pablo UMAÑA
Signature of first Inventor:	Date:
Residence:	
Citizenship:	
Mailing Address:	

Full name of second Inventor:	Joël JEAN-MAIRET
Signature of second Inventor:	Date:
Residence:	
Citizenship:	
Mailing Address:	

Full name of third Inventor:	James E. BAILEY (deceased), signed by M. Sean BAILEY as legal representative
Signature of legal representative of third Inventor:	 Date: 8/11/06
Residence of legal representative, M. Sean BAILEY:	Santa Monica, CA
Citizenship:	UNITED STATES
Mailing Address:	591 Entrada Drive Santa Monica, CA 90402

# Getting the glycosylation right: Implications for the biotechnology industry

Nigel Jenkins\*, Raj B. Parekh<sup>1</sup>, and David C. James<sup>2</sup>

<sup>1</sup>Department of Biological Sciences, De Montfort University, Leicester LE1 9BH, UK. <sup>2</sup>Oxford Glycoscience, Hitching Court, Blacklands Way, Abingdon OX14 1RG, UK. <sup>1</sup>Research School of Biosciences, University of Kent, Canterbury, Kent CT2 7NJ, UK. \*Corresponding author (e-mail: njenkins@dmu.ac.uk).

Received 4 March 1996; accepted 18 June 1996.

Glycosylation is the most extensive of all the posttranslational modifications, and has important functions in the secretion, antigenicity and clearance of glycoproteins. In recent years major advances have been made in the cloning of glycosyltransferase enzymes, in understanding the varied biological functions of carbohydrates, and in the accurate analysis of glycoprotein heterogeneity. In this review we discuss the impact of these advances on the choice of a recombinant host cell line, in optimizing cell culture processes, and in choosing the appropriate level of glycosylation analysis for each stage of product development.

Keywords: glycosylation, recombinant, antigenicity, clearance, review, carbohydrates

better understanding of glycoprotein biosynthetic pathways and oning of many of the key enzymes involved, together with rogress in assigning functions to specific carbohydrate structures, as provided the groundwork for exploitation of glycobiology ithin the biotechnology industry. The carbohydrate components f glycoproteins can play crucial roles in protein folding, oligomer assembly and secretion processes, and in the clearance of glycoproteins from the bloodstream. Certain carbohydrate structures ave also been found to be antigenic, and regulatory authorities such as the US Food and Drug Administration are demanding increasingly sophisticated carbohydrate analyses as part of the product or process validation<sup>1,2</sup>.

It is not the purpose of this review to provide a general update n advances in glycobiology, which have already been covered by several excellent reviews published on biosynthetic pathways<sup>3-6</sup>, ie biological properties of carbohydrates<sup>7</sup>, and cellular influences n the glycosylation process<sup>8-11</sup>. Instead, this review will focus on the differences in carbohydrate structures that may arise from choosing alternative gene expression systems and culture conditions, their physiological significance, and the level of carbohydrate analysis that is appropriate at each stage of product evelopment.

## Oligosaccharide structures found on glycoproteins

Oligosaccharides can attach to proteins in three ways (Fig.1): (1) Via an N-glycosidic bond to the R-group of an Asn residue within the consensus sequence Asn-X-Ser/Thr (N-glycosylation). All mature N-linked glycan structures have a common core of GlcNAc, which can form part of simple oligomannose structures or be extensively modified by the addition of other residues such as fucose, galactose, and sialic acids. Hybrid structures also exist where one or more arms of the glycan are modified and the remaining arm(s) contain only mannose. (2) Via an O-glycosidic

bond to the R-group of Ser or Thr (O-glycosylation). O-linked glycosylation is extensive in structural proteins such as proteoglycans. Small glycan structures can also be O-linked to the side chain of hydroxylsine or hydroxyproline. (3) Carbohydrates are also components of the glycoprophosphatidylinositol anchor used to secure some proteins to cell membranes.

The presence of these consensus sequences by no means guarantees their glycosylation. They show varying degrees of occupancy with oligosaccharides (macroheterogeneity) dependent on their position within the protein and its conformation, the host cell type used for expression, and its physiological status. These three factors also determine the extent of variation in the type of sugar residues found within each oligosaccharide (microheterogeneity).

## Choice of expression system

**Bacteria.** Common bacterial expression systems such as *Escherichia coli* have no capacity to glycosylate proteins in either N- or O-linked conformations<sup>12</sup>. Although other bacterial strains such as *Neisseria meningitidis* have recently been shown to O-glycosylate certain of their endogenous proteins, the trisaccharide added is different from O-linked sugars found in eukaryotes<sup>13</sup>.

**Yeast.** Hypermannosylation (the addition of a large number of mannose residues to the core oligosaccharide), is a common property of most yeast strains<sup>14</sup> and can compromise the efficacy of recombinant proteins such as the hepatitis B vaccine<sup>15</sup> (Table 1). But hypermannosylation can be prevented by expressing the polypeptide in mutant yeast strains (e.g., mnn-9 or the temperature-sensitive ngd-29) in which N-glycosylation is confined to core oligosaccharide residues with a limited mannose content (up to Man<sub>3</sub>GlcNAc<sub>2</sub>), resulting in more effective vaccines<sup>13,16</sup>. There is also evidence to suggest that different O-glycosylation sites are used by yeast and mammalian cells<sup>17</sup>.

## REVIEW ARTICLE

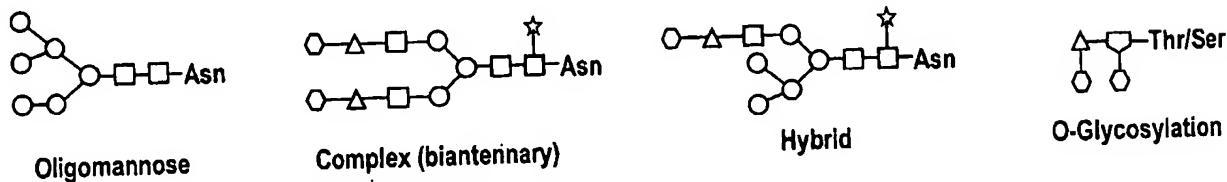


Figure 1. Common oligosaccharide structures found on glycoproteins: O Man, □ GlcNAc, △ Gal, ○ NeuAc, ★ Fucose, ▽ GalNAc.

**Plants.** The few studies reporting the production of human proteins in plants have suggested that simple *N*-glycan structures that lack sialic acids are added, which may compromise activity. For example, erythropoietin (EPO) produced in tobacco cells has no biological activity *in vivo*, presumably because of its high clearance rate<sup>18</sup>. Another obstacle may be the presence of potentially allergenic residues such as core  $\alpha$ 1-3 linked fucose<sup>19</sup> or xylose  $\beta$ 1-2 mannose<sup>20</sup>.

**Insects.** The baculovirus-infected insect cell expression system has become a popular route for recombinant protein synthesis because of its short process development time and potentially high yields. Most evidence to date indicates that the *N*-glycosylation capabilities of this system are limited to producing only simple oligomannose-type oligosaccharides ( $\text{Man}_{3-5}\text{GlcNAc}_2$ )<sup>21-23</sup>; only a few studies have demonstrated complex *N*-linked glycans<sup>24</sup>. However, most of these data are derived from *Spodoptera frugiperda* (Sf9 and Sf21 lines), and other baculovirus-infected insect cell lines may differ. For example, the Ea4 line (derived from *Estigmene acrea*) can add some terminal galactose residues to recombinant interferon- $\gamma$ <sup>25</sup>, and secreted alkaline phosphatase produced in *Trichoplusia ni* (TN-368 and BTI-Tn-5B1-4 lines) has

both galactose and terminal sialic acids<sup>26</sup>.

**Mammals.** Species that are phylogenetically closer to humans may be expected to have more elements of the glycosylation machinery in common. Nevertheless, there are some surprising differences between the glycosylation characteristics of the rodent cell lines (routinely used for recombinant glycoprotein synthesis) and human tissues.

**Mouse cells.** Most mammals express the enzyme  $\alpha$ 1-3-galactosyltransferase, which generates  $\text{Gal}\alpha 1,3\text{Gal}\beta 1,4\text{GlcNAc}$  residues on membrane and secreted glycoproteins. The notable exceptions are in humans, apes, and Old World monkeys where the gene has become inactivated through frameshift mutations<sup>27</sup>. Certain mouse lines such as hybridomas, mouse-human heterohybridomas, and C127 cells synthesize some glycans terminating in  $\text{Gal}\alpha 1,3\text{Gal}\beta 1,4\text{GlcNAc}$ <sup>28,29</sup>, particularly when grown in nonagitated flasks<sup>20,30</sup>. But other rodent lines such as mouse NS0 or rat Y0 myeloma, producing humanized antibodies, do not add  $\text{Gal}\alpha 1,3\text{Gal}\beta 1,4\text{GlcNAc}$ <sup>31,32</sup>, and only induce a mild human immune response<sup>33</sup>. These residues are more likely to occur in hybridoma-derived antibodies where glycosylation occurs in the light chain, compared to the partially buried Fc glycosylation site<sup>34</sup>. Over 1% of

Table 1. Summary of the major glycosylation attributes for different cell expression systems.

Organism	Cell type	Type of Glycosylation			Saccharide Residues									
		O-linked	Oligo-mannose	Hyper-mannose	Complex	Fucose	Galactose	Sialic acids	Bisecting	Glyco-				
						$\alpha$ 1-6 linked	$\alpha$ 1-3 linked	Gal $\alpha$ 1-3 Gal	SO <sub>4</sub> -GalNAc	$\alpha$ 2,6 linked	$\alpha$ 2,3 linked	NeuGc	GlcNAc	Glyco-sidas
Bacterium	<i>E. coli</i>	0	0	0	0	0	0	0	0	0	0	0	0	?
Yeast	<i>Sacromyces</i>	++	0	++++	0	0	0	0	0	0	0	0	0	?
Plant	Tobacco BY2	?	++	0	?	++	?	0	0	0	0	0	0	++
Insect	<i>S. frugiperda</i> Sf9	++	++++	0	D	++	+	0	0	0	0	0	0	?
	<i>S. frugiperda</i> Sf21	++	++++	0	D	++	?	?	?	?	D	D	?	0
	<i>T. ni</i>	?	++	0	++	?	?	?	?	?	++	++	?	0
	<i>E. acrea</i> Ea4	?	+++	0	++	++	0	0	0	0	0	0	0	?
	<i>M. brassicae</i>	++	++++	0	0	++	?	?	?	?	?	?	0	?
Hamster	BHK	++	++	0	++	++	0	D	0	0	0	++	+	++
	CHO	++	++	0	++	++	0	++	0	0	0	++	++	0
Mouse	Hybridoma	++	++	0	++	++	0	++	0	+	+	++	++	+
	Myeloma	++	++	0	++	++	0	++	++	++	++	++	++	0
	C127	++	++	0	++	++	0	++	?	++	++	++	++	?
	J558L	++	?	0	++	++	0	++	?	?	+	+	?	?
Rat	Transgenic	++	++	0	++	++	0	?	?	?	+	+	?	?
Goat & Sheep	Y0 Myeloma	?	?	0	++	?	0	?	?	?	+	+	?	?
Human	Transgenic	?	++	0	++	++	0	?	?	?	+	+	?	?
	Liver	++	+	0	++	++	0	0	0	++	++	0	0	?
	Brain	++	++	0	++	++	++	0	0	++	++	0	0	?
	Pituitary	++	++	0	++	++	0	0	++	+	+	0	++	?
	B-lymphocyte	++	0	0	+	++	0	0	0	+	+	0	++	?
	Nanawha	++	++	0	++	++	0	0	0	++	++	?	?	?
Human-Mouse	Heterohybridoma	?	++	0	++	++	0	0	0	+	+	+	0	?

Note that a few studies have analyzed the glycosylation profile of the same glycoprotein expressed in more than one system, therefore some characteristics may be protein-specific. Further details of individual cell lines are given in the text. 0=not detected; ?=not tested; D=disputed (conflicting results reported in different publications). + to ++++: an approximation of the levels of oligosaccharides detected.

human serum IgG is directed against the Gal(α1,3)-Gal-β1,4-GlcNAc epitope<sup>16</sup>, which may be a consequence of its presence on enteric bacteria. Specific removal of this epitope from porcine endothelial cells substantially diminishes their reaction with the natural cytotoxic antibodies found in human serum<sup>17</sup>, but comparisons of mouse C127 and Chinese hamster ovary (CHO)-derived tissue plasminogen activator (tPA) reveal only minor differences in the pharmacokinetics induced by interaction with these antibodies<sup>18</sup>.

N-glycolylneuraminic acid (NeuGc) is a derivative of the sialic acid N-acetylneuraminic acid (NeuAc), and NeuGc levels have been shown to be more prevalent than NeuAc in antibodies derived from mouse or human-mouse hybridomas<sup>19</sup>. In contrast, glycoproteins in adult humans do not normally contain NeuGc, which is an oncofetal antigen<sup>20</sup>. Low levels of NeuGc (1% of total sialic acids) are tolerated in recombinant proteins such as EPO, but higher levels (e.g., in fetuin containing 7% NeuGc) can elicit an anti-NeuGc immune response<sup>21</sup>. Furthermore, high levels of terminal NeuGc on a chimeric CT4-IgG fusion protein are correlated with a rapid removal of the molecule from circulation, compared to the same protein bearing terminal NeuAc residues<sup>22</sup>. The hydroxylase enzyme that converts cytidine 5'-phosphate (CMP)-NeuAc to CMP-NeuGc has recently been cloned<sup>23</sup>.

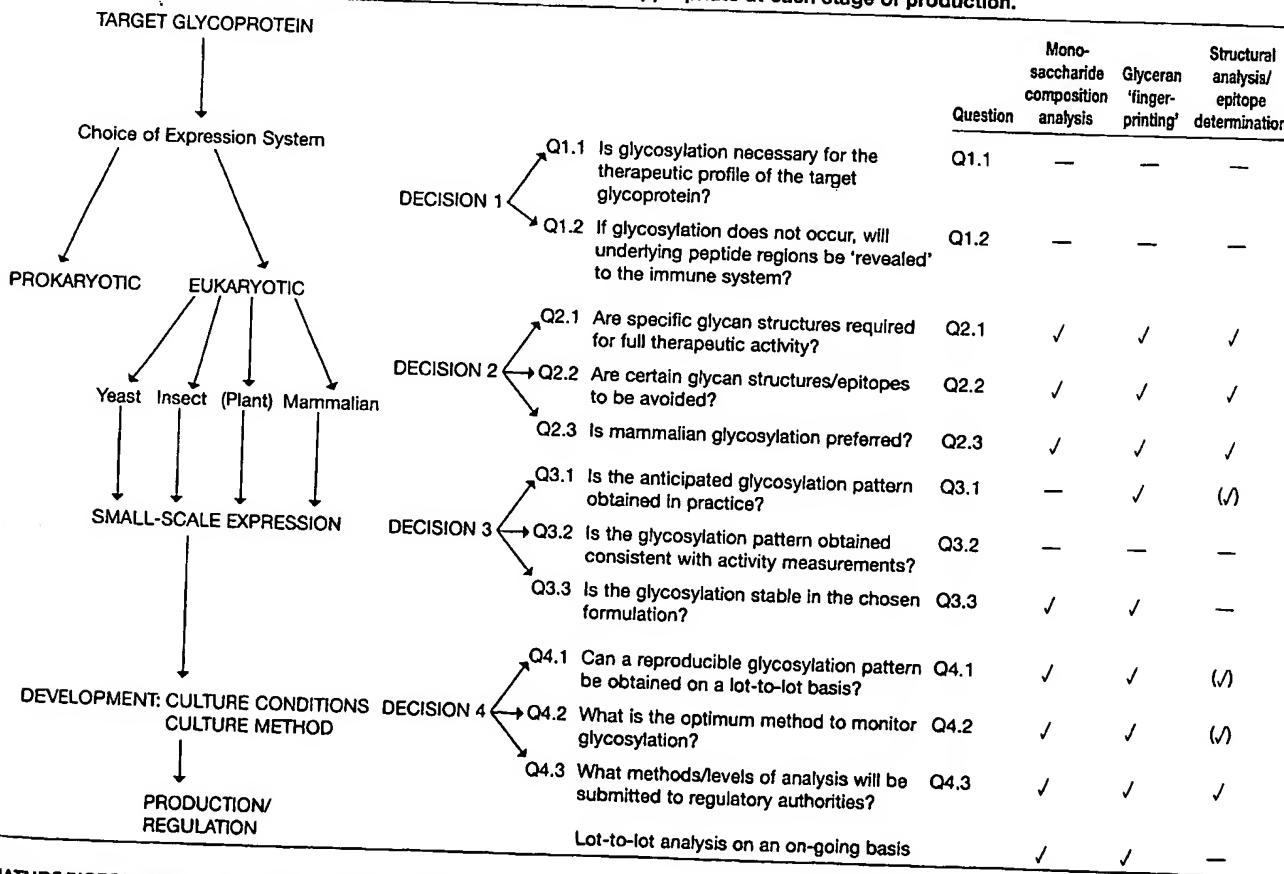
**Hamster cells.** Most of the CHO cell lines used for recombinant protein synthesis, such as Dux-B11, have fortuitously inactivated the gene for α1-3-galactosyltransferase<sup>24</sup> and make low levels of NeuGc (NeuAc is the dominant sialic acid found on CHO-derived glycoproteins)<sup>25</sup>. But cell lines such as CHO and baby hamster kidney (BHK) lack a functional α2,6-sialyltransferase

(ST) enzyme, and synthesize exclusively α2,3-linked terminal sialic acids via α2,3-ST, in contrast to human and mouse cells, which have both enzymes<sup>26</sup>. Most rodent cells, including the CHO cell line, can be genetically modified to resemble the human glycan profile by transfection of appropriate glycosyltransferases. For example, the gene for rat α2,6-ST has been cloned into a recombinant CHO cell line to produce glycoproteins with both α2,6-linked and α2,3-linked NeuAc<sup>25-27</sup>. In addition, many mutants of CHO cells that display altered glycosylation properties that may prove useful hosts for expressing glycoproteins with minimal heterogeneity have been isolated<sup>28</sup>.

**Human cells.** Other host cell lines have not been studied in sufficient depth to precisely define their glycosylation capabilities. Even the use of human host cell lines is not perfect, since the transformation event required in most cases to produce a stable cell line may itself result in altered glycosylation profiles<sup>29</sup>. However, the human lymphoblastoid Namalwa cell line performs O-linked and N-linked glycosylation efficiently, and preliminary studies on Namalwa-derived recombinant tPA have demonstrated all the human-type glycosylation characteristics<sup>26,31</sup>. All mouse-human heterohybridomas examined thus far have been found to follow the glycosylation characteristics of the mouse parental line<sup>26,32</sup>.

**Transgenic animals.** Relatively few studies have been reported on the glycosylation of recombinant proteins expressed in the milk of transgenic animals. Those published from experiments in transgenic goats<sup>33</sup> suggest that a low level of complex glycans may be achieved. Furthermore, interferon-γ (IFN-γ) expressed in transgenic mice showed a greater proportion of truncated and oligomannose structures at the Asn<sub>n</sub> site compared to IFN-γ that

**Table 2. Decisions governing the type of glycosylation analyses appropriate at each stage of production.**



## REVIEW ARTICLE

expressed in CHO cells, although the level of glycosylation site occupancy was increased<sup>21</sup>. Glycoproteins can be remodelled in situ by the transgenic expression of extra glycosyltransferases in the mouse mammary gland<sup>22</sup>.

**Cells derived from specialized tissues.** A significant proportion of IgG molecules produced by human B-lymphocytes possess a bisecting GlcNAc residue  $\beta$ 1-4 linked to the central  $\beta$ -linked mannose of the core glycan. This residue may play a role in antibody-dependent cell-mediated cytotoxicity, and only certain rodent cell lines such as the rat YO myeloma (but not CHO cells or NS0 myelomas) produce recombinant antibodies containing this bisecting residue<sup>23</sup>. The GlcNAc transferase III enzyme which adds the residue has been cloned, thus, the opportunity exists for remodelling chimeric antibodies by transfecting this gene into host cells<sup>24</sup>.

A large body of evidence suggests that natural IgG molecules lacking galactose are associated with rheumatoid arthritis<sup>25</sup>. This has also been tested experimentally by presenting the agalactosyl glycoforms of autoantibodies recognizing type II collagen in T-cell-primed mice, resulting in acute synovitis<sup>26</sup>. Thus, expression systems that result in a large proportion of recombinant therapeutic antibodies lacking galactose should be avoided. Low levels of sialic acids are typically found in both recombinant and natural antibodies<sup>27</sup>, probably due to steric hindrance at the Fc glycosylation site.

Sulfated residues ( $\text{SO}_4\text{-GalNAc-GlcNAc}$ ) appear on the outer arms of glycans attached to several pituitary glycoprotein hormones such as luteinizing hormone (LH), thyrotropin, pro-opiomelanocortin and certain proteases such as urokinase<sup>28,29</sup>. A specific GalNAc transferase and a terminal GalNAc sulfotransferase recognize special protein motifs in the nascent peptide (e.g., Pro-Leu-Arg) and are mainly restricted to the anterior pituitary gland. Therefore, only cell lines derived from the pituitary gland or endothelium (such as At20 and 293 cells) are able to perform this sulfation<sup>30,31</sup>. A hepatic receptor binds oligosaccharides terminating with  $\text{SO}_4\text{-GalNAc}$  residues, and could account for the rapid removal of sulfated LH from the circulation in contrast to the removal of follicle-stimulating hormone and chorionic gonadotropin, which bear terminal NeuAc residues<sup>32</sup>. Mouse C127 cells produce a different pattern of sulfation (NeuAc- $\alpha$ 3SO<sub>4</sub>-6Gal), which occurs after the addition of NeuAc<sup>33</sup>.

The clearance of a given glycoprotein from the blood stream is highly dependent on its oligosaccharides, particularly those that are situated on the outer arms of the glycan structures<sup>34</sup>. Indeed, artificial glycosylation sites have even been introduced into small peptides in order to improve their pharmacokinetic properties or to render them resistant to blood proteases<sup>35,36</sup>. There are several receptors for specific oligosaccharide structures that contribute to the clearance of glycoproteins from the bloodstream, the most significant being the asialoglycoprotein receptor<sup>37</sup>. Other binding proteins recognize specific structures such as the Man- $\alpha$ 6Man- $\beta$ 4GlcNAc- $\beta$ 4GlcNAc found in interleukin-6 (IL-6)<sup>38</sup>, but their significance in the clearance of the majority of glycoproteins remains to be determined<sup>39</sup>. Studies in rats using EPO fractions enriched for particular glycoforms indicate that more highly branched glycans (e.g., triantennary or tetraantennary) are less susceptible to renal clearance than biantennary structures<sup>40</sup>.

### Cell culture conditions

Once the host cell line has been chosen, the cell culture conditions should be optimized in order to minimize glycoprotein heterogeneity and prevent deterioration of product quality during fermentation. Pioneering studies analyzing glycoproteins from batch samples<sup>20,21</sup> demonstrated that the culture environment can influence both the macroheterogeneity and microheterogeneity of

oligosaccharides in recombinant glycoproteins<sup>19,21,22</sup>.

**Cell culture media.** Monoclonal IgG, produced by mouse hybridomas in serum-free medium has higher levels of terminal sialic acid and galactose residues relative to that produced using serum<sup>21</sup>, but antibody galactosylation is better in serum-containing medium for recombinant CHO cells<sup>23</sup>. Production of antibodies in cell culture results in more consistent glycosylation than achieved in ascites fluid<sup>19,22,23</sup>. Adaptation of BHK-21 cells producing a recombinant IL-2 mutant, from serum-containing to serum-free medium, results in substantial changes to its glycosylation, such as the complexity of glycan chains (number of arms, and higher levels of terminal sialylation and proximal  $\alpha$ 1-6 fucosylation), and increases the overall level of glycosylation<sup>24</sup>.

The ambient glucose concentration affects the degree of glycosylation of monoclonal antibodies produced by human hybridomas in batch culture<sup>25</sup>, and of IFN- $\gamma$  produced by CHO cells in continuous culture<sup>26,27</sup>. Lipids such as dolichol act as key carriers in the glycosylation process, and lipid supplements alone or in combination with lipoprotein carriers can improve the N-glycosylation site occupancy of IFN- $\gamma$ <sup>28,29</sup>. Work on hepatocytes suggests that provision of cytidine and uridine can also alter protein glycosylation by increasing the availability of nucleotide-sugars<sup>30</sup>.

The application of sophisticated analytical techniques to analyze glycosylation microheterogeneity has led to some interesting findings. An increase in the percentage of oligomannose (predominantly Man<sub>n</sub>GlcNAc<sub>n</sub>) and truncated structures has been observed when monitoring both recombinant antibodies made by NS0 myelomas<sup>28,31</sup> and IFN- $\gamma$  made by recombinant CHO cells<sup>21</sup>. In the former study, supplemental nutrients in fed-batch culture did not prevent this deterioration in glycosylation. In perfusion culture of BHK-21 cells producing a recombinant IL-2 mutant, nutrient limitations (glucose, amino acids, dO<sub>2</sub>) led to short-term changes in macroheterogeneity, but microheterogeneity was largely unchanged<sup>24</sup>.

**Cell status.** Lowering the protein synthetic rate by cycloheximide improves the glycosylation site occupancy of recombinant prolactin produced by C127 cells<sup>32</sup>, but studies on tPA synthesis in CHO cells suggest that the rate of protein synthesis by itself has little influence on protein glycosylation<sup>33</sup>. Folding and disulfide bond formation certainly can influence the efficiency of N-linked glycosylation in some proteins. For example, low concentrations of the reducing agent dithiothreitol prevent cotranslational disulfide bond formation in the endoplasmic reticulum and lead to complete glycosylation of a tPA sequon that normally undergoes variable glycosylation<sup>34</sup>. Cell growth rate influences glycosyltransferase V levels in HepG2 cells<sup>35</sup>. Sodium butyrate is sometimes used to improve protein synthesis, but can change glycosylation by inducing a GlcNAc-transferase involved in O-glycosylation<sup>36</sup> and increasing sialyltransferase activity in recombinant CHO cells<sup>37,38</sup>. (Curiously, butyrate has the opposite effect in HepG2 cells<sup>39</sup>.)

**Bioreactor configuration.** Mild hypoxia has minimal effects on the glycosylation of tPA produced by recombinant CHO cells<sup>32</sup>, but influences the level of sialylation of recombinant FSH<sup>40</sup>. Similarly pH changes within the range 6.9–8.2 in the cell culture medium do not have a dramatic effect on the glycosylation profile of recombinant placental lactogen expressed in CHO cells, however there was some evidence for underglycosylation outside this range<sup>41</sup>.

Increases in the concentration of ammonium ion in the culture medium above 2 mM may compromise sialyltransferase present in the Golgi, resulting in reduced  $\alpha$ 2,6-linked sialic acid in G-CSF produced by recombinant CHO cells<sup>42</sup>. Increases in ammonium ions also reduced the extent of recombinant placental lactogen N-glycosylation by CHO cells, but this was dependent on the pH<sup>43</sup>.

**Product degradation.** Various glycosidase activities have been measured in CHO cell lysates and culture supernatants, the most active of which is sialidase at neutral pH<sup>103,104</sup>. The soluble enzyme has been purified from culture supernatant of CHO cells<sup>104</sup> and can degrade glycans from proteins such as recombinant gp120. The CHO cell gene coding for this soluble sialidase was subsequently cloned and showed structural similarities to bacterial sialidases<sup>105</sup>. Although CHO cells also produce an  $\alpha$ -L-fucosidase, this enzyme is incapable of releasing core  $\alpha$ 1,6-fucose from intact recombinant glycoproteins (gp120 or CD4), or the more peripheral Fuc- $\alpha$ 1,3-GlcNAc linkage from serum  $\alpha$ -acid glycoprotein<sup>106</sup>. Sialidase,  $\beta$ -galactosidase,  $\beta$ -hexosaminidase, and fucosidase can be detected at low levels in supernatants from mouse 293, NS0, and hybridoma cells, and the sialidase activity is much lower than that found in CHO cells<sup>101</sup>.

In a contrasting study, purified monoclonal antibody was incubated with supernatants from various mammalian cell lines (CHO K1, BHK-21, mouse C127, P3-X63, Ag8.653, and a human-mouse heterohybridoma) and an insect cell line (SF-21AE), and only the insect line showed evidence of glycosidase activity in the supernatant<sup>107</sup>.

**Controlled carbohydrate modification.** Advances are being made in the chemical production of oligosaccharides (e.g., synthesis of the core N-glycan structure Man<sub>3</sub>GlcNAc<sub>2</sub> and oligomannose glycans from monosaccharides has recently been accomplished<sup>108,109</sup>). This opens the possibility of adding defined glycan structures after recombinant protein synthesis and secretion. In addition, the cloning and expression in *E. coli* of several key glycosyltransferases will facilitate the postharvest remodelling of glycoproteins produced in cell culture<sup>105,106</sup>, making them more acceptable as human therapeutic proteins.

## Conclusions

Improvements in glycosylation analysis have enabled scientists to judge how the glycan structures of recombinant glycoproteins compare to their natural human counterparts. Ultimately, production may go beyond the reiteration of the human glycosylation profile, because it may be desirable to alter the product's bioactivity or pharmacokinetics in vivo by altering specific glycan structures. The initial choice of expression system will continue to be of crucial importance, and as more recombinant proteins are expressed in different cell lines a pattern of glycoform predictions can be assembled (Table 1), although these conclusions must remain speculative until more data are available. The influences of the cell culture process (including the effects of scale-up) are not as well defined at present, but as more studies are published generic protocols may emerge to produce consistent (albeit heterogeneous) glycosylation patterns. A more difficult objective for cell technology will be to produce a single invariant protein glycoform, rather than the current mixture of glycoforms each bearing different characteristics (analogous to chiral separation of small molecule drugs).

An increased awareness of the importance of product glycosylation has led to more detailed carbohydrate analysis at earlier stages of product development than in the past. The choice of a particular cell expression system, and the influence of a different manufacturing process on the biochemical consistency of the product are now being evaluated (Table 2). A small but increasing number of glycosylation analyses are requested by legal departments, who may use the resulting information in patent applications to substantiate claims and to distinguish their organization's material from that of their competitors.

## Acknowledgments

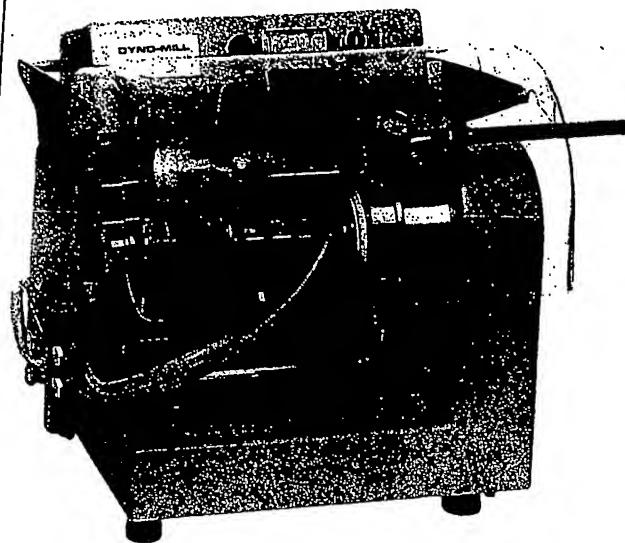
I and DCJ are supported by grants from the Biotechnology and Biological Research Council of the UK.

1. Gelsow, M.J. 1992. Glycoprotein glycans—roles and controls. *Trends Biotechnol.* 10:333-335.
2. Liu, D.T.Y. 1992. Glycoprotein pharmaceuticals—scientific and regulatory considerations, and the United-States Orphan Drug-Act. *Trends Biotechnol.* 10:114-120.
3. Abeijon, C. and Hirschberg, C.B. 1992. Topography of glycosylation reactions in the endoplasmic reticulum. *Trends Biochem. Sci.* 17:32-36.
4. Hayes, B.K. and Hart, G.W. 1994. Novel forms of protein glycosylation. *Curr. Opinion Struct. Biol.* 4:692-696.
5. Ziozias, D.H. 1992. Mammalian glycosyltransferases: genomic organization and protein structure. *Glycobiology* 2:271-277.
6. Field, M.C. and Wainwright, L.J. 1995. Molecular-cloning of eukaryotic glycoprotein and glycolipid glycosyltransferases—a survey. *Glycobiology* 5:463-472.
7. Varki, A. 1993. Biological roles of oligosaccharides: all of the theories are correct. *Glycobiology* 3:97-130.
8. Parekh, R.B. and Patel, T.P. 1992. Comparing the glycosylation patterns of recombinant glycoproteins. *Trends Biotechnol.* 10:276-280.
9. Jenkins, N. and Curling, E.M. 1994. Glycosylation of recombinant proteins: problems and prospects. *Enzyme Microb. Technol.* 16:354-364.
10. Goochee, C.F. and Monica, T.J. 1990. Environmental effects on protein glycosylation. *Bio/Technology* 8:421-427.
11. Goochee, C.F., Gramer, M.J., Andersen, D.C., Bahr, J.B., and Rasmussen, J.R. 1991. The oligosaccharides of glycoproteins—bioprocess factors affecting oligosaccharide structure and their effect on glycoprotein properties. *Bio/Technology* 9:1347-1355.
12. Letourneau, O., Sechi, S., Willettebrown, J., Robertson, M.W., and Kinet, J.P. 1995. Glycosylation of human truncated Fc-epsilon-RI  $\alpha$ -chain is necessary for efficient folding in the endoplasmic-reticulum. *J. Biol. Chem.* 270:8249-8256.
13. Stimson, E., Virji, M., Makepeace, K., Dell, A., Morris, H.R., Payne, G., et al. 1995. Meningococcal pilin—a glycoprotein substituted with digalactosyl 2,4-diacetamido-2,4,6-trideoxyhexose. *Mol. Microbiol.* 17:1201-1214.
14. Herscovics, A.O. and Orlean, P. 1993. Glycoprotein biosynthesis in yeast. *FASEB J.* 7:540-550.
15. Kniskern, P.J., Hagopian, A., Burke, P., Schulz, L.D., Montgomery, D.L., Hurni, W.M., et al. 1994. Characterization and evaluation of a recombinant hepatitis-B vaccine expressed in yeast defective for N-linked hyperglycosylation. *Vaccine* 12:1021-1025.
16. Lehle, L., Eiden, A., Lehnert, K., Haselbeck, A., and Kopetzki, E. 1995. Glycoprotein-biosynthesis in *Saccharomyces cerevisiae*—ngd29, an N-glycosylation mutant allelic to och1 having a defect in the initiation of outer chain formation. *FEBS Lett.* 370:41-45.
17. Kalsner, I., Schneider, F.J., Geyer, R., Ahorn, H., and Maurefogy, I. 1992. Comparison of the carbohydrate moieties of recombinant soluble Fc-epsilon receptor (sFc- $\epsilon$ -RII/scd23) expressed in *Saccharomyces cerevisiae* and Chinese hamster ovary cells—different O-glycosylation sites are used by yeast and mammalian cells. *Glycoconjugate J.* 9:209-216.
18. Matsumoto, S., Ikura, K., Ueda, M., and Sasaki, R. 1995. Characterization of a human glycoprotein (erythropoietin) produced in cultured tobacco cells. *Plant Mol. Biol.* 27:1163-1172.
19. Altmann, F., Tretter, V., Kubelka, V., Staudacher, E., Marz, L., and Becker, W.M. 1993. Fucose in  $\alpha$ 1-3 linkage to the N-glycan core forms an allergenic epitope that occurs in plant and in insect glycoproteins. *Glycoconjugate J.* 10:301.
20. Ma, J.K.C. and Hein, M.B. 1995. Immunotherapeutic potential of antibodies produced in plants. *Trends Biotechnol.* 13:522-527.
21. James, D.C., Freedman, R.B., Hoare, M., Ogonah, O.W., Rooney, B.C., Larionov, O.A., et al. 1995. N-glycosylation of recombinant human interferon- $\gamma$  produced in different animal expression systems. *Bio/Technology* 13:592-596.
22. Gruberhorst, E., Hofer, B., Nlmitz, M., Jager, V., and Conradi, H.S. 1993. Biosynthesis and secretion of human interleukin-2 glycoprotein variants from baculovirus-infected sf21 cells—characterization of polypeptides and post-translational modifications. *Eur. J. Biochem.* 215:189-197.
23. Jarvis, D.L. and Finn, E.E. 1995. Biochemical-analysis of the N-glycosylation pathway in baculovirus-infected lepidopteran insect cells. *Virology* 212:500-511.
24. Davidson, D.J.C., Fraser, M.J., and Castellino, F.J. 1990. Oligosaccharide processing in the expression of human plasminogen cDNA by lepidopteran insect (*Spodoptera frugiperda*) cells. *Biochemistry* 29:5584-5590.
25. Ogonah, O.W., Freedman, R.B., Jenkins, N., Patel, K., and Rooney, B.C. 1996. Isolation and characterization of an insect-cell line able to perform complex N-linked glycosylation on recombinant proteins. *Bio/Technology* 14:197-202.
26. Davis, T.R. and Wood, H.A. 1995. Intrinsic glycosylation potentials of insect-cell cultures and insect larvae. *In Vitro Cellular Develop. Biol.-Animal* 31:659-663.
27. Larsen, R.D., Rivera-Marrero, C.A., Ernst, L.K., Cummings, R.D., and Lowe, J.B. 1990. Frameshift and nonsense mutations in a human genomic sequence homologous to a murine UDP-Gal:G-D-Gal(1,4)-D-GlcNAc  $\alpha$ (1,3)-galactosyltransferase cDNA. *J. Biol. Chem.* 265:7055-7061.
28. Borrebaek, C.A.K., Malmborg, A.C., and Ohlin, M. 1993. Does endogenous glycosylation prevent the use of mouse monoclonal antibodies as cancer therapeutics? *Immunol. Today* 14:477-479.
29. Tsuji, J., Norma, S., Suzuki, J., Okumura, K., and Shimizu, N. 1990. Specificity of human natural antibody to recombinant tissue-type plasminogen activator (t-PA) expressed in mouse C127 cells. *Chem. Pharm. Bull.* 38:765-768.
30. Lund, J.T., Takahashi, N., Hindley, S.A., Tyler, R., Goodall, M., and Jefferts, R. 1993. Glycosylation of human IgG subclass and mouse IgG2b heavy chains secreted by mouse JS58L transfecoma cell lines as chimeric antibodies. *Human Antibodies & Hybridsomas* 4:20-25.
31. Lund, J.T., Takahashi, N., Nakagawa, H., Goodall, M., Bentley, T., Hindley, S.A., et al. 1993. Control of IgG/Fc glycosylation: a comparison of oligosaccharides from chimeric human/mouse and mouse subclass immunoglobulin Gs. *Mol. Immunol.* 30:741-748.

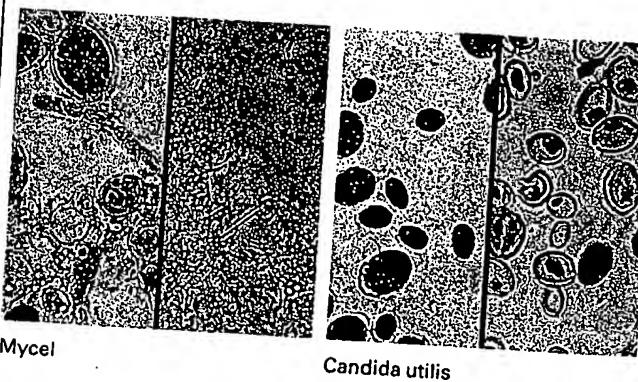
## REVIEW ARTICLE

81. Robinson, D.K., Chan, C.P., Ip, C.Y., Tsai, P.K., Tung, J., Seamans, T.C., et al. 1994. Characterization of a recombinant antibody produced in the course of a high-yield fed-batch process. *Biotechnol. Bioeng.* 44:727-735.
82. Hooker, A.D., Goldman, M.H., Markham, N.H., James, D.C., Ison, A.P., Bull, A.T., et al. 1995. N-glycans of recombinant human interferon- $\gamma$  change during batch culture of Chinese-hamster ovary cells. *Biotechnol. Bioeng.* 48:639-648.
83. Gawlik, M., Conradt, H.S., and Wagner, R. 1995. Effect of different cell-culture conditions on the polypeptide integrity and N-glycosylation of a recombinant model glycoprotein. *Biotechnol. Bioeng.* 46:536-544.
84. Shelikoff, M., Sinskey, A.J., and Stephanopoulos, G. 1994. The effect of protein-synthesis inhibitors on the glycosylation site occupancy of recombinant human prolactin. *Cytotechnology* 15:195-208.
85. Buleid, N.J., Bassel-Duby, R.S., Freedman, R.B., Sambrook, J.F., and Gethling, M.J. 1992. Cell-free synthesis of enzymically active tissue-type plasminogen activator. Protein folding determines the extent of N-linked glycosylation. *Biochem. J.* 286:275-280.
86. Allen, S., Naim, H.Y., and Buleid, N.J. 1995. Intracellular folding of tissue-type plasminogen-activator—effects of disulfide bond formation on N-linked glycosylation and secretion. *J. Biol. Chem.* 270:4797-4804.
87. Hahn, T.J. and Goochee, C.F. 1992. Growth-associated glycosylation of transferrin secreted by HepG2 cells. *J. Biol. Chem.* 267:23982-23987.
88. Datti, A. and Dennis, J.W. 1993. Regulation of UDP-GlcNAc:Gal B 1-3GalNAc transferase 1-6-N-acetylglucosaminyltransferase (GlcNAc to GalNAc) in Chinese hamster ovary cells. *J. Biol. Chem.* 268:5409-5416.
89. Chotigat, W., Watanapokesin, Y., Mahler, S., and Gray, P.P. 1994. Role of environmental-conditions on the expression levels, glycoform pattern and levels of sialyltransferase for hFSH produced by recombinant CHO cells. *Cytotechnology* 15:217-221.
90. Gebert, C.A. and Gray, P.P. 1995. Expression of FSH in CHO cells .2. stimulation of hFSH expression levels by defined medium supplements. *Cytotechnology* 17:13-19.
91. Shah, S., Lance, P., Smith, T.J., Berenson, C.S., Cohen, S.A., Horvath, P.J., et al. 1992. N-butyrate reduces the expression of  $\beta$ -galactosidase  $\alpha$ 2,6-sialyltransferase in Hep G2 cells. *J. Biol. Chem.* 267:10652-10658.
92. Lin, A.A., Kimura, R., and Miller, W.M. 1993. Production of tPA in recombinant CHO cells under oxygen-limited conditions. *Biotechnol. Bioeng.* 42:339-350.
93. Borys, M.C., Linzer, D.J.H., and Papoutsakis, E.T. 1993. Culture pH affects expression rates and glycosylation of recombinant mouse placental lactogen proteins by Chinese hamster ovary (CHO) cells. *Bio/Technology* 11:720-724.
94. Andersen, D.C., Goochee, C.F., Cooper, G., and Weitzhandler, M. 1994. Monosaccharide and oligosaccharide analysis of isoelectric focusing-separated and blotted granulocyte-colony-stimulating factor glycoforms using high-pH anion-exchange chromatography with pulsed amperometric detection. *Glycobiology* 4:459-467.
95. Borys, M.C., Linzer, D.J.H., and Papoutsakis, E.T. 1994. Ammonia affects the glycosylation patterns of recombinant mouse placental lactogen-1 by Chinese hamster ovary cells in a pH-dependent manner. *Biotechnol. Bioeng.* 43:505-514.
96. Gramer, M.J. and Goochee, C.F. 1993. Glycosidase activities in Chinese hamster ovary cell lysate and cell-culture supernatant. *Biotechnol. Prog.* 9:366-373.
97. Gramer, M.J., Goochee, C.F., Chock, V., Brousseau, D.T., and Sliwkowski, M.B. 1995. Removal of sialic acid from a glycoprotein in CHO cell supernatant by action of an extracellular CHO cell sialidase. *Bio/Technology* 13:692-698.
98. Warner, T.G., Chang, J., Ferrari, J., Harris, R., McNealey, T., Bennett, G., et al. 1993. Isolation and properties of a soluble sialidase from the culture fluid of Chinese-hamster ovary cells. *Glycobiology* 3:455-463.
99. Ferrari, J., Harris, R., and Warner, T.G. 1994. Cloning and expression of a soluble sialidase from Chinese-hamster ovary cells—sequence alignment similarities to bacterial sialidases. *Glycobiology* 4:367-373.
100. Gramer, M.J., Schaffer, D.V., Sliwkowski, M.B., and Goochee, C.F. 1994. Purification and characterization of  $\alpha$ -L-fucosidase from Chinese-hamster ovary cell-culture supernatant. *Glycobiology* 4:611-616.
101. Gramer, M.J. and Goochee, C.F. 1994. Glycosidase activities of the 293 and NSO cell-lines, and of an antibody-producing hybridoma cell-line. *Biotechnol. Bioeng.* 43:423-428.
102. Ackermann, M., Marx, U., and Jager, V. 1995. Influence of cell-derived and media-derived factors on the integrity of a human monoclonal antibody after secretion into serum-free cell-culture supernatants. *Biotechnol. Bioeng.* 45:97-106.
103. Matsuo, I., Nakahara, Y., Ito, Y., Nukada, T., and Ogawa, T. 1995. Synthesis of a glycopeptide carrying a N-linked core pentasaccharide. *Bio-organic & Med. Chem.* 3:1455-1463.
104. Nakahara, Y., Shibayama, S., and Ogawa, T. 1996. Rationally designed synthesis of high-mannose and complex type undecasaccharides. *Carbohydr. Res.* 280:67-84.
105. Hermann, G.F., Ichikawa, Y., Wandrey, C., Gaeta, F.C.A., Paulson, J.C., and Wong, C.H. 1993. A new multienzyme system for a one-pot synthesis of sialyl oligosaccharides—combined use of  $\beta$ -galactosidase and  $\alpha$ (2, 6)-sialyltransferase coupled with regeneration in situ of CMP-sialic acid. *Tetrahedron Lett.* 34:3091-3094.
106. Galli, U. and Anaraki, F. 1995.  $\alpha$ -galactosyl (Gal $\alpha$ 1- $\alpha$ 1-3Gal-1-4GlcNAc-1) epitopes on human cells—synthesis of the epitope on human red-cells by recombinant primate  $\alpha$ 1,3-galactosyltransferase expressed in *Escherichia coli*. *Glycobiology* 5:775-782.

# DYNO®-MILL



DYNO-MILL Type KDL 600 ml



### DYNO® Laboratory Mill Type KDL

for the

- desintegration of microorganisms
- finest wet milling and dispersion of pharmaceutical products and products of the chemical food industry etc.

in research and production.

Horizontal working ball mill for *continuous and possibly batch operation* of very fluid up to very viscous suspensions which can be pumped in a completely closed system. All parts coming into contact with the suspension and the grinding containers in different sizes are easily changeable. Optimal disintegration of microbiological material (yeast, mycel, algae, bacteria) in shortest time at a low temperature.



SINCE 1933

**Willy A. Bachofen AG Maschinenfabrik**

CH-4005 Basel/Schweiz, Utengasse 15/17  
Phone 061/6815151, Telex 962564 wab ch, Fax (061) 6815058

READER INQUIRY NO. 320

## REVIEW ARTICLE

32. Yu-Ip, C.C., Miller, W.J., Silberklang, M., Mark, G.E., Ellis, R.W., Huang, L.H., et al. 1994. Structural characterization of the N-glycans of a humanized anti-CD8 murine immunoglobulin G. *Arch. Biochem. Biophys.* 308:387-399.
33. Lifely, M.R., Hale, C., Boyce, S., Keen, M.J., and Phillips, J. 1995. Glycosylation and biological activity of CAMPATH-1H expressed in different cell lines and grown under different culture conditions. *Glycobiology* 5:813-822.
34. Stephens, S., Emtage, S., Vetterlein, O., Chaplin, L., Bebbington, C., Nesbitt, A., et al. 1995. Comprehensive pharmacokinetics of a humanized antibody and analysis of residual anti-idiotypic responses. *Immunology* 85:668-674.
35. Endo, T., Wright, A., Morrison, S.L., and Kobata, A. 1995. Glycosylation of the variable region of immunoglobulin G: site-specific maturation of the sugar chains. *Mol. Immunol.* 32:931-940.
36. Hamadeh, R.M., Jarvis, G.A., Galili, U., Mandrell, R.E., Zhou, P., and Griffiss, J.M. 1992. Human natural anti-Gal IgG regulates alternative complement pathway activation on bacterial surfaces. *J. Clin. Invest.* 89:1223-1235.
37. Lavecchia, J.A., Dunne, A.D., and Edge, A.S.B. 1995. Enzymatic removal of  $\alpha$ -galactosyl epitopes from porcine endothelial cells diminishes the cytotoxic effect of natural antibodies. *Transplantation* 60:841-847.
38. Monica, T.J., Williams, S.B., Goochee, C.F., and Maiorella, B.L. 1995. Characterization of the glycosylation of a human-IgM produced by a human-mouse hybridoma. *Glycobiology* 5:175-185.
39. Muchmore, E.A., Milewski, M., Varki, A., and Diaz, S. 1989. Biosynthesis of N-glycolyneuraminic acid. The primary site of hydroxylation of N-acetylneurameric acid is the cytosolic sugar nucleotide pool. *J. Biol. Chem.* 264:20216-20223.
40. Noguchi, A., Mukuria, C.J., Suzuki, E., and Naiki, M. 1995. Immunogenicity of N-glycolyneuraminic acid-containing carbohydrate chains of recombinant human erythropoietin expressed in Chinese hamster ovary cells. *J. Biochem.* 117:59-62.
41. Flesher, A.R., Marzowski, J., Wang, W.C., and Raff, H.V. 1995. Fluorophore labeled glycan analysis of immunoglobulin fusion proteins: correlation of oligosaccharide content with in vivo clearance profile. *Biotechnol. Bioeng.* 46:399-407.
42. Kawano, T., Koyama, S., Takematsu, H., Kozutsumi, Y., Kawasaki, H., Kawashima, S., et al. 1995. Molecular cloning of cytidine monophospho-N-acetylneurameric acid hydroxylase: regulation of species-specific and tissue-specific expression of N-glycolyneuraminic acid. *J. Biol. Chem.* 270:16458-16463.
43. Smith, D.F., Larsen, R.D., Mattox, S.A., Lowe, J.B., and Cummings, R.D. 1990. Transfer and expression of a murine UDP-Gal:3'-D-Gal $\alpha$ 1,3-galactosyltransferase gene in transfected Chinese hamster ovary cells. Competition reactions between the  $\alpha$ 1,3-galactosyltransferase and the endogenous  $\alpha$ 2,3-sialyltransferase. *J. Biol. Chem.* 265:6225-6234.
44. Hokke, C.H., Bergwerff, A.A., Vandendriessche, G.W.K., Kamerling, J.P., and Vliegenthart, J.F.G. 1995. Structural analysis of the sialylated N-linked and O-linked carbohydrate chains of recombinant human-erythropoietin expressed in Chinese hamster ovary cells—sialylation patterns and branch location of dimeric N-acetyllactosamine units. *Eur. J. Biochem.* 228:981-1008.
45. Lee, E.U., Roth, J., and Paulson, J.C. 1989. Alteration of terminal glycosylation sequences on N-linked oligosaccharides of Chinese hamster ovary cells by expression of B-galactosidase  $\alpha$ 2,6-sialyltransferase. *J. Biol. Chem.* 264:13848-13855.
46. Minch, S.L., Kallio, P.T., and Bailey, J.E. 1995. Tissue-plasminogen activator co-expressed in Chinese hamster ovary cells with  $\alpha$ 2,6-sialyltransferase contains NeuAc $\alpha$ 2,6Gal $\beta$ 1,4GlcNAc linkages. *Biotechnol. Prog.* 11:348-351.
47. Grabenhorst, E., Hoffmann, A., Nimitz, M., Zettmeissl, G., and Conradt, H.S. 1995. Construction of stable BHK-21-cells coexpressing human secretory glycoproteins and human Gal(3-1)4GlcNAc-r  $\alpha$ -2,6-sialyltransferase  $\alpha$ -2,6-linked NeuAc is preferentially attached to the Gal(3-1)4GlcNAc(8-1-2)Man(6-1-3)-branch of diantennary oligosaccharides from secreted recombinant  $\beta$ -trace protein. *Eur. J. Biochem.* 232:718-725.
48. Stanley, P. and Ioffe, E. 1995. Glycosyltransferase mutants—key to new insights in glycobiology. *FASEB J.* 9:1436-1444.
49. Yamashita, K., Koide, N., Endo, T., Iwaki, Y., and Kobata, A. 1989. Altered glycosylation of serum transferin of patients with hepatocellular carcinoma. *J. Biol. Chem.* 264:2415-2423.
50. Khan, M.W., Musgrave, S.C., and Jenkins, N. 1995. N-linked glycosylation of tissue-plasminogen activator in Namalwa cells. *Biochem. Soc. Trans.* 23:S99.
51. Okamoto, M., Nakai, M., Nakayama, C., Yanagi, H., Matsui, H., Noguchi, H., et al. 1991. Purification and characterization of three forms of differently glycosylated recombinant human granulocyte-macrophage colony-stimulating factor. *Arch. Biochem. Biophys.* 286:562-568.
52. Tandai, M., Endo, T., Sasaki, S., Masuho, Y., Kochibe, N., and Kobata, A. 1991. Structural study of the sugar moieties of monoclonal antibodies secreted by human-mouse hybridoma. *Arch. Biochem. Biophys.* 291:339-348.
53. Cole, E.S., Higgins, E., Bernasconi, R., Garone, L., and Edmunds, T. 1994. Glycosylation patterns of human proteins expressed in transgenic goat milk. *J. Cell Biochem.* 265:S18D.
54. Prieto, P.A., Mukerji, P., Kelder, B., Erney, R., Gonzalez, D., Yun, J.S., et al. 1995. Remodeling of mouse milk glycoconjugates by transgenic expression of a human glycosyltransferase. *J. Biol. Chem.* 270:29515-29519.
55. Miyoshi, E., Ihara, Y., Hayashi, N., Fusamoto, H., Kamada, T., and Taniguchi, N. 1995. Transfection of N-acetylglucosaminyltransferase-III gene suppresses expression of hepatitis-B virus in a human hepatoma-cell line, HB611. *J. Biol. Chem.* 270:28311-28315.
56. Newkirk, M.M., Fournier, M.J., and Shiroky, J. 1995. Rheumatoid-factor avidity in patients with rheumatoid arthritis—identification of pathogenic RFs which correlate with disease parameters and with the Gal $\beta$  glycoform of IgG. *J. Clin. Immunol.* 15:250-257.
57. Rademacher, T.W., Williams, P., and Dwek, R.A. 1994. Agalactosyl glycoforms of IgG autoantibodies are pathogenic. *Proc. Natl. Acad. Sci. USA* 91:6123-6127.
58. Siciliano, R.A., Morris, H.R., Bennett, H.P.J., and Dell, A. 1994. O-glycosylation mimics N-glycosylation in the 16-kDa fragment of bovine pro- $\alpha$ -melanocortin—the major O-glycan attached to Thr45 carries SO $\beta$ -4GalNAc-81,4GlcNAc-8-1, which is the archetypal nonreducing epitope in the N-glycans of pituitary glycohormones. *J. Biol. Chem.* 269:910-920.
59. Bergwerff, A.A., Van Oosterom, J., Kamerling, J.P., and Vliegenthart, J.F.G. 1995. The major N-linked carbohydrate chains from human urokinase—the occurrence of 4-O-sulfated, ( $\alpha$ 2,6)-sialylated or ( $\alpha$ -1,3)-fucosylated N-acetylgalactosamine(6-1-4)-N-acetylglucosamine elements. *Eur. J. Biochem.* 228:1009-1019.
60. Skelton, T.P., Kumar, S., Smith, P.L., Beranek, M.C., and Baenziger, J.U. 1992. Pro- $\alpha$ -melanocortin synthesized by corticotrophs bears asparagine-linked oligosaccharides terminating with SO $\beta$ -4GalNAc-81,4GlcNAc-8-1,2Man  $\alpha$ . *J. Biol. Chem.* 267:12998-13006.
61. Smith, P.L. and Baenziger, J.U. 1992. Molecular basis of recognition by the glycoprotein hormone-specific N-acetylgalactosamine-transferase. *Proc. Natl. Acad. Sci. USA* 89:329-333.
62. Flete, D., Srivastava, V., Hindsgaul, O., and Baenziger, J.U. 1991. A hepatic reticuloendothelial cell receptor specific for SO $\beta$ -4GalNAc-81,4GlcNAc-8-1,2Man that mediates rapid clearance of lutropin. *Cell* 67:1103-1110.
63. Pfeiffer, G., Strube, K.H., and Geyer, R. 1992. Biosynthesis of sulfated glycoprotein-N-glycans present in recombinant human tissue plasminogen activator. *Biochem. Biophys. Res. Commun.* 189:1681-1685.
64. Takei, Y., Chiba, T., Wada, K., Hayashi, H., Yamada, M., Kuwashima, J., et al. 1995. Glycosylated human recombinant interleukin-1- $\alpha$ , neo interleukin-1- $\alpha$ , with d-mannose dimer exhibits selective activities in-vivo. *J. Interferon & Cytokine Res.* 15:713-719.
65. Baudys, M., Uchio, T., Hovgaard, L., Zhu, E.F., Avramoglou, T., Jozefowicz, M., et al. 1995. Glycosylated insulins. *J. Controlled Release* 38:151-157.
66. Monroe, R.S. and Huber, B.E. 1994. The major form of the murine asialoglycoprotein receptor—cDNA sequence and expression in liver, testis and epididymis. *Gene* 148:237-244.
67. Parekh, R.B., Dwek, R.A., Rademacher, T.W., Opdenakker, G., and Vandamme, J. 1992. Glycosylation of interleukin-6 purified from normal human blood mononuclear cells. *Eur. J. Biochem.* 203:135-141.
68. Drickamer, K. 1991. Clearing up glycoprotein hormones. *Cell* 67:1029-1032.
69. Misrahi, T., Matsu, S., Strickland, T.W., Takeuchi, M., Kobata, A., and Takasaki, S. 1995. Role of antennary structure of N-linked sugar chains in renal handling of recombinant human erythropoietin. *Blood* 86:4097-4104.
70. Spellman, M.W., Basa, L.J., Leonard, C.K., Chakel, J.A., O'Connor, J.V., Wilson, S. et al. 1989. Carbohydrate structures of human tissue plasminogen activator expressed in Chinese hamster ovary cells. *J. Biol. Chem.* 264:14100-14111.
71. Hotchkiss, A., Refino, C.J., Leonard, C.K., O'Connor, J.V., Crowley, C., McCabe, J., et al. 1988. The influence of carbohydrate structure on the clearance of recombinant tissue-type plasminogen-activator. *Thromb. Haemost.* 60:255-261.
72. Patel, T.P., Parekh, R.B., Moellering, B.J., and Prior, C.P. 1992. Different culture methods lead to differences in glycosylation of a murine IgG monoclonal antibody. *Biochem. J.* 285:839-845.
73. Maiorella, B.L., Winkefahke, J., Young, J., Moyer, B., Bauer, R., Hora, M., et al. 1993. Effect of culture conditions on IgM antibody structure, pharmacokinetics and activity. *BioTechnology* 11:387-392.
74. Gawlik, M., Valley, U., Nimitz, M., Wagner, R., and Conradt, H.S. 1995. Characterization of changes in the glycosylation pattern of recombinant proteins from BHK-21-cells due to different culture conditions. *J. Biotechnol.* 42:117-131.
75. Tachibana, H., Taniguchi, K., Ushio, Y., Teruya, K., Osada, K., and Murakami, H. 1994. Changes of monosaccharide availability of human hybridoma lead to alteration of biological properties of human monoclonal antibody. *Cytotechnology* 16:151-157.
76. Hayter, P.M., Curling, E.M., Gould, M.L., Baines, A.J., Jenkins, N., Salmon, I., et al. 1993. The effect of dilution rate on CHO cell physiology and recombinant interferon- $\gamma$  production in glucose-limited chemostat cultures. *Biotechnol. Bioeng.* 39:327-335.
77. Hayter, P.M., Curling, E.M., Baines, A.J., Jenkins, N., Salmon, I., Strange, P.G., et al. 1992. Glucose-limited chemostat culture of Chinese hamster ovary cells producing recombinant human interferon- $\gamma$ . *Biotechnol. Bioeng.* 39:327-335.
78. Castro, P.M.L., Ison, A.P., Hayter, P.M., and Bull, A.T. 1995. The macroheterogeneity of recombinant human interferon- $\gamma$  produced by Chinese hamster ovary cells is affected by the protein and lipid content of the culture medium. *Biotechnol. Appl. Biochem.* 27:87-100.
79. Jenkins, N., Castro, P.M.L., Menon, S., Ison, A.P., and Bull, A.T. 1994. Effect of lipid supplements on the production and glycosylation of recombinant interferon- $\gamma$  expressed in CHO cells. *Cytotechnology* 15:209-215.
80. Rijcken, W.R.P., Overdijk, B., Vandeneijnden, D.H., and Ferwerda, W. 1995. The effect of increasing nucleotide sugar concentrations on the incorporation of sugars into glycoconjugates in rat hepatocytes. *Biochem. J.* 305:865-870.

# nature biotechnology

VOLUME 14 NUMBER 8 • AUGUST 1996

FORMERLY BIOTECHNOLOGY

A  
tr  
irus-mediated  
nesis

200002 APR 97 02063 5:91617 20005  
SKEESLER, AFTN LIBRARY BI 0028  
GOLD, FOX  
AT&T LIBRARY 600  
NEW YORK AVE  
WASHINGTON DC 20005-3934



**Chilling effects  
of biolipid  
engineering**

**Nitric oxide  
detection and  
imaging *in vivo***

**Disabling shade  
avoidance to improve  
crop growth**

**SPECIAL  
SECTION**  
INTERNATIONAL  
GUIDE TO  
REGIONAL  
DEVELOPMENT

## CONTENTS



Nature America Inc.

**NEW YORK**  
Nature America, Inc.  
345 Park Avenue South  
New York, NY 10010-1707  
Tel: +1 (212) 726-9200  
Fax: +1 (212) 696-9006

*President and Publisher*  
Mary Waltham

*Vice President Advertising*  
Marion Delaney

*Vice President Marketing*  
Jim Skovrenski

*Production and I.S. Director*  
Niki Kemp

*Advertising Sales Manager*  
William C. Moran  
(New York)  
Kathryn Wayman (London)

*Classified Advertising Sales Manager*  
Iain Jawad (London)  
Miriam Kirraline  
(New York)

*Marketing Manager*  
Edelyn Enero (New York)  
Nic Harman (London)

*Assistant Marketing Manager*  
Carolyn Blenkin (London)  
Thomas Mathew  
(New York)

*Production Manager*  
Estelle Seizer

*WWW Project Manager*  
Mark Goodstein  
(New York)  
*m.goodstein@natureny.com*

**LONDON**  
Macmillan Magazines, Ltd.  
Porters South  
Crinan Street  
London N1 9XW  
Tel: +44 171 833 4000  
Fax: +44 171 843 4996

*Managing Director*  
Ray Barker

*Publishing Director*  
Andy Sutherland

*Editor-in-Chief (Nature publications)*  
Philip Campbell

*Art Director*  
Jane Walker

**TOKYO**  
Nature Japan KK  
Shin Mitsuke Building  
3-8 Ichigaya-Tamachi  
Shinjuku, Tokyo 162  
Tele: +81 3 3267 8751  
Fax: +81 3 3267 8746

*Publisher*  
David Swinbanks

*Manager*  
Koichi Nakamura

*Advertising Sales Manager*  
Philip Hamill

*Marketing Manager*  
Richard Nathan

## Research News



The adenovirus and the egg: A new approach to transgenesis .....	942
Alan R. Clarke	
From curse to cure: HIV for gene therapy? .....	943
Michael Emerman	
NO NMDA receptor activity .....	944
David S. Bredt	
A new vision for plant productivity.....	945
Peter H. Quail	
Designer oils for better nutrition .....	946
Anthony J. Kinney	
Metal in restrained means.....	947
Andy Morby	
Taking DNA probes into a protein world .....	947
Sanjay Tyagi	
Adenoviruses get safe, effective, and specific .....	948
James Kling	

## SPECIAL SECTION: REGIONAL DEVELOPMENT 1996



South American starter cultures .....	951
Rafael Rangel-Aldao	
Quebec biotechnology: The quiet revolution .....	955
Alden Finch Prouty	
Partnering in Japan.....	958
Ted T. Tanaka	
Biotechnology in Southeast Asia.....	960
Deborah Miller	
The Caledonian way .....	962
Niamh Quinn	
Switzerland: At a turning point .....	964
Olivier Binet	
Wisconsin-grown biotechnology .....	965
Richard Burgess	

An international listing of regional development organizations .....

## RESEARCH REVIEW

Getting the glycosylation right: Implications for the biotechnology industry .....	975
Nigel Jenkins, Raj B. Parekh, and David C. James	

## RESEARCH ARTICLES



Transgenesis by adenovirus-mediated gene transfer into mouse zona-free eggs .....	982
Tohru Tsukui, Yumi Kanegae, Izumu Saito, and Yutaka Toyoda	



Nature Biotechnology (ISSN 1087-0156), formerly Bio/Technology (ISSN 0733-2220), is published monthly, with a special issue in November, by Nature Publishing Co., headquartered at 345 Park Avenue South, New York, NY 10010-1707, which is owned by Nature America Inc., a subsidiary of Macmillan Magazines Ltd., of London. North American advertising: Nature Biotechnology, 345 Park Avenue South, New York, NY 10010-1707; telephone +1 (212) 726-9200; fax +1 (212) 696-9006. European advertising: Nature Biotechnology, Porters South, Crinan Street, London N1 9SQ; telephone +44 (171) 833 4000; fax +44 (171) 843 4996. New subscriptions, renewals, changes of address, back issues, and all customer service questions should be addressed to: Nature Biotechnology subscription department, P.O. Box 5054, Brentwood, TN 37024-5054, or call toll-free U.S./Canada/Mexico: +1 (800) 524-0328. Outside North America: Nature Biotechnology circulation manager, Macmillan Magazines, Brunel Road, Basingstoke, Hampshire RG212XS, U.K. Annual subscription rates: £149 (individual making personal payment); Rest of world (excluding Japan): £200 (institutional/corporate), U.S. \$59, Canada add 7% for GST (individual making personal payment); U.K./Europe: £180 (institutional/corporate), £149 (individual making personal payment); Rest of world (excluding Japan): £200 (institutional/corporate), U.S. \$59, Canada add 7% for GST (individual making personal payment); Japan: Contact Japan Publications Trading Co., Ltd., 2-1 Serugaku-cho 1 chome, Chiyoda-ku, Tokyo 101, Japan, phone (03) 292-3755. Back Issues: U.S./Canada: \$40, Canada add 7% for GST; Rest of world: surface U.S. \$43, air mail U.S. \$45. Reader Inquiry service: Nature Biotechnology, Nature America Inc., Reader Service Department, P.O. Box 5484, Pittsfield, MA 01203-8332. Reprints: Nature Biotechnology telnet to [copher.internet.com](http://copher.internet.com) and log-in as *gnnews*, and the Nature homepage at [www.nature.com](http://www.nature.com). POSTMASTER: Send address changes to Nature Biotechnology subscription department, P.O. Box 5054, Brentwood, TN 37024-5054. Periodicals postage paid at New York, NY 10010-1707 and additional mailing offices. Executive Officers of Nature America, Inc.: Nicholas Byam Shaw, chairman of the board; Mary Waltham, president; Edward Valls, secretary-treasurer. Copyright © Nature America, Inc.



# nature biotechnology

FORMERLY BIO/TECHNOLOGY

VOLUME 14 AUGUST 1996

*The cover. Section of a mouse embryo expressing an adenovirus-mediated lacZ transgene, shown as ubiquitous blue staining. Details can be found on pp. 942 and 982.*

**Editor**  
Susan Hassler  
(New York)

**Managing Editor**  
Andrew Marshall  
(New York)

**Editor-at-Large**  
Harvey Blay  
(New York)

**Associate Research Editor**  
Philip Bernstein  
(New York)

**Senior Editors**  
Stephen M. Edgington  
(New York)  
John Hodgson  
(London)

**Assistant Editor**  
Emma Dorey  
(New York)

**Editorial Assistants**  
Michael Francisco  
(New York)  
Emma Johnson  
(London)

**Intern**  
Tara Marathe

**Contributing Writers**  
Bernard Dixon  
Jeffrey L. Fox  
Vicki Glaser  
Russ Hoyle  
Mike Ward

**Associate Art Director**  
Renée M. Roberts  
(New York)

**NEW YORK**  
Editorial Office  
Nature America, Inc.  
345 Park Avenue South  
New York, NY 10010-1707  
Tel: +1 (212) 726-9200  
Fax: +1 (212) 686-9006  
m.francisco@natureny.com

**LONDON**  
Macmillan Magazines, Ltd.  
Porters South  
Cranham Street  
London N1 9XW  
Tel: +44 171 833 4000  
Fax: +44 171 843 4998  
e.johnson@biotechnology.com

**WORLD WIDE WEB**  
biotech.nature.com

## OPINION

### Editorial

Biodiamonds and paying back debts ..... 925

### Commentary

Did George do the right thing? ..... 926  
Stephen M. Edgington

US national bioethics commission: Politics as usual? ..... 927  
Russ Hoyle

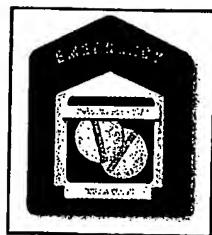
UK government blows a hollow trumpet ..... 928  
Mike Ward and John Hodgson

Reconstructing the biochemical edifice ..... 928  
William Bains

### Correspondence

Cassava R&D: A public investment ..... 929

## IN BRIEF



### Business and Regulatory News

MS drug trials cast light on oral tolerance mechanisms... New sources of funding: a hair-raising prospect?... Initial public offerings... "Morning after" pills approved before NDA filing... Immune suppressor drug companies forge ahead... Dithymidine for that "sensible" tan... Shareholders don't put much stock in home improvement... Gene-of-the-month club... Preventing blood clots with Streptomyces... UK dominates European biotech... Joint ventures ..... 930

### Nature Biotechnology Research

NMDA receptor antagonists... NO imaging... Sandwich oligo assay... Stabilized streptavidin... Metal scavengers... Microinjection alternative... Chilling resistance... Novel ion exchange ..... 933

## ANALYSIS

### Business and Regulatory News

Public companies spend over \$4 billion on R&D ..... 934  
Clinical costs escalate biotechnology's R&D expenses ..... 937  
Genentech and Mitsubishi unblock stroke market ..... 939  
Honeymoon not yet over for pharma-biotech deals ..... 940  
Pathogen patchwork could cultivate noncompliance ..... 940  
Safer pertussis vaccines for adult use ..... 941

## CONTENTS

**Display Representatives**  
**US East Coast**  
Eric Gordon  
+1 (800) 988-1112

**US Midwest**  
John D. Carr  
Steve Loerch  
+1 (847) 498-4520

**US West Coast & Southwest**  
Lynne Stickrod  
Heidi Y. Gregory  
+1 (800) 229-9758

**United Kingdom, Finland, Denmark**  
Kathryn Wayman  
Sheila Davitt  
+44 (171) 843-4960

**Southern Germany & Austria**  
Sabine Furst  
+49 (89) 52-40-81

**Northern Germany**  
Andrea Küchler  
+49 (40) 3600-137

**Scandinavia**  
Alexandra Hewardt  
+46 (8) 644-0005

**France/Holland/Belgium**  
Virginie Déletreaz  
+33 (14) 387-4217

**Switzerland**  
Verena Loewenthal  
+41 (52) 761 3030

**Japan**  
Philip Hamill  
+81 (3) 267-8751

**Australia**  
Anne Murray  
+61 (2) 299-5877

**Scientific Advisers**  
Ken-ichi Arai  
Univ. Tokyo  
Roger Beachy  
Scripps Res. Inst.  
Teruhiko Beppu  
Nihon University  
Ronald E. Care  
Dowling Molecular  
Jean-Pierre Changeux  
Inst. Pasteur  
Mary-Dell Chilton  
CIBA-Geigy  
Nam-Hai Chua  
Rockefeller Univ.  
Rita R. Colwell  
Maryland Biotechnology Inst.  
Arnold Demain  
MIT

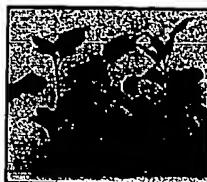
David Goeddel  
Tutank  
Leroy Hood  
Univ. Washington, Seattle  
Morio Ikenbara  
Protein Engineering Res. Inst.  
Ernest Jaworski  
Monsanto Co.  
Kary Mullis  
Consultant  
Victor Nussenzweig  
NY Univ. Med. Ctr.

Gregory Petsko  
Brandeis Univ.  
George Poste  
SmithKline Beecham  
George Rose  
Johns Hopkins Univ. Sch. Med.

Carl-Gustaf Rosen  
Abitec AB  
Kendall Smith  
NY Univ./Cornell Med. Ctr.  
Yukio Sugino  
Takoda Chemicals  
Marc Van Montagu  
Univ. Ghent  
Indra K. Vasil  
Univ. Florida  
Wataru Yamaya  
Seikagaku Kogyo  
Douglas C. Young  
KAIROS Scientific Inc.

**In vitro selection of peptides acting at a new site of NMDA glutamate receptors** ..... 986  
Min Li, Weifeng Yu, Chien-Huan Chen, Steve Cwirla, Erik Whitehorn, Emily Tate, Ron Raab,  
Meire Bremer, and Bill Dower

**In vivo EPR detection and imaging of endogenous nitric oxide in lipopolysaccharide-treated mice** ..... 992  
Tetsuhiko Yoshimura, Hidekatsu Yokoyama, Satoshi Fujii, Fusako Takayama,  
Kazuo Oikawa, and Hitoshi Kamada



**Genetic engineering of harvest index in tobacco through overexpression of a phytochrome gene** ..... 995  
Paul R.H. Robson, Alex C. McCormac, Anne S. Irvine, and Harry Smith

**The thermodynamic control of protein binding to lipid bilayers for protein chromatography** ..... 999  
Angelika Loidl-Stahlhofen, Stefan Kaufmann, Thomas Braunschweig, and Thomas M. Bayerl

**Low-temperature resistance of higher plants is significantly enhanced by a nonspecific cyanobacterial desaturase** ..... 1003  
Osamu Ishizaki-Nishizawa, Toshio Fujii, Mizue Azuma, Keiko Sekiguchi, Norio Murata,  
Takeshi Ohtani, and Toshihiro Toguri

**Streptavidins with intersubunit crosslinks have enhanced stability** ..... 1007  
Gabriel O. Reznik, Sandor Vajda, Cassandra L. Smith, Charles R. Cantor, and Takeshi Sano



**A method to codetect introduced genes and their products in gene therapy protocols** ..... 1012  
Emanuela Gussoni, Yaming Wang, Cornel Fraefel, Robert G. Miller,  
Helen M. Blau, Alfred I. Geller, and Louis M. Kunkel

**Enhanced metaladsorption of bacterial cells displaying poly-His peptides** ..... 1017  
Carolina Sousa, Angel Cebolla, and Víctor de Lorenzo

**An enzyme-linked oligonucleotide assay** ..... 1021  
Daniel W. Drolet, Lotus Moon-McDermott, and Timothy S. Romig

## RESOURCES

### Patents

**Patent pitfalls among the unpredictable arts** ..... 1028  
Brian P. O'Shaughnessy

**Some recent patent applications in the area of combinatorial libraries** ..... 1029

### New Products

**Clean rooms** ..... 1030

### WWW Guide

**Combinatorial libraries on the Web** ..... 1031  
Richard Peters and Robert S. Sikorski

### People

**Promotions, advancements** ..... 1031

## CLASSIFIED

**Market section** ..... 1039

**Employment opportunities, meetings, symposia** ..... 1040

REVIEW/SYNTHÈSE

## Biosynthetic controls that determine the branching and microheterogeneity of protein-bound oligosaccharides<sup>1</sup>

HARRY SCHACHTER

Research Institute, Hospital for Sick Children, Toronto, Ont., Canada

and

Department of Biochemistry, University of Toronto, Toronto, Ont., Canada

Received October 18, 1985

Schachter, H. (1986) Biosynthetic controls that determine the branching and microheterogeneity of protein-bound oligosaccharides. *Biochem. Cell Biol.* 64, 163-181

Detailed studies on the enzyme machinery responsible for the biosynthesis of protein-bound oligosaccharides of the Asn-GlcNAc and Ser(Thr)-GalNAc linkage types have allowed the formulation of some general rules which explain, at least in part, the branching patterns and microheterogeneity of these structures. These rules are discussed under the following headings: (i) competition of two or more enzymes for a common substrate; (ii) controls at the level of enzyme substrate specificity (e.g., critical sugar residues which turn enzyme activity on or off, branch specificity, and the role of the polypeptide in the glycoprotein substrate); (iii) substrate availability.

Schachter, H. (1986) Biosynthetic controls that determine the branching and microheterogeneity of protein-bound oligosaccharides. *Biochem. Cell Biol.* 64, 163-181

Les études détaillées de la machinerie enzymatique responsable de la biosynthèse des oligosaccharides liés aux protéines par des liaisons du type Asn-GlcNAc et Ser(Thr)-GalNAc ont permis la formulation de certaines règles générales qui expliquent, du moins en partie, les profils de ramifications et la microhétérogénéité de ces structures. Nous discutons de ces règles sous les rubriques suivantes : (i) compétition de deux enzymes ou plus pour un substrat commun; (ii) contrôles au niveau de la spécificité enzymatique à l'égard des substrats (p. ex., résidus glucidiques critiques qui amorcent ou qui arrêtent l'activité enzymatique, spécificité des ramifications, rôle du polypeptide dans le substrat glycoprotéique); (iii) disponibilité des substrats.

[Traduit par la revue]

### Introduction

Protein-bound oligosaccharides fall into several classes depending on the linkage between sugar and amino acid. The classes to be discussed in this review are of the Asn-GlcNAc (*N*-glycosidic linkage) and Ser(Thr)-GalNAc (*O*-glycosidic linkage) types. Modern analytical techniques have recently led to the elucidation of literally hundreds of different protein-bound oligosaccharides. This work has shown that two properties distinguish complex carbohydrates from proteins and nucleic acids, the other two classes of biological macromolecules, namely (i) branching and (ii) microheterogeneity. These properties pose both challenges

and problems for researchers interested in the function and biosynthesis of complex carbohydrates.

The functions of complex carbohydrates are not properly understood and will not be discussed in detail. Several different functions have been identified (1). (i) The presence or absence of different oligosaccharides can modulate the physicochemical properties of a glycoprotein, e.g., solubility, viscosity, charge, and denaturation. (ii) Carbohydrate may protect the polypeptide moiety of a glycoprotein against uncontrolled proteolysis, both inside and outside the cell. (iii) Some glycoproteins undergo proteolytic processing within the cell from a large primary translation product to smaller final products. This process can be affected by the presence of protein-bound carbohydrate. (iv) Although carbohydrate is not usually involved in the biological activity of biologically active glycoproteins, there are important exceptions, e.g., human chorionic gonadotropin. (v) Some glycoproteins require proper glycosylation for their insertion into membrane, for secretion, or for proper intracellular migration and sorting. (vi) Glycosylation has been implicated in embryonic development and differentiation.

We are only beginning to understand some of these

ABBREVIATIONS: Gn or GlcNAc, *N*-acetylglucosamine; GN or GalNAc, *N*-acetylgalactosamine; (Gn), bisecting GlcNAc; G, or Gal, galactose; M or Man, mannose; F or Fuc, fucose; S, sialic acid; Gn-T or GlcNAc-T, *N*-acetylglucosaminyltransferase; G-T or Gal-T, galactosyltransferase;  $\alpha$ Mase II,  $\alpha$ -mannosidase II; F-T or Fuc-T, fucosyltransferase; S-T or sialyl-T, sialyltransferase; IgG, immunoglobulin G; H, heavy; L, light; NMR, nuclear magnetic resonance.

<sup>1</sup>This talk was given at the presentation of the Boehringer Mannheim Canada Prize of the Canadian Biochemical Society, on June 18, 1985.

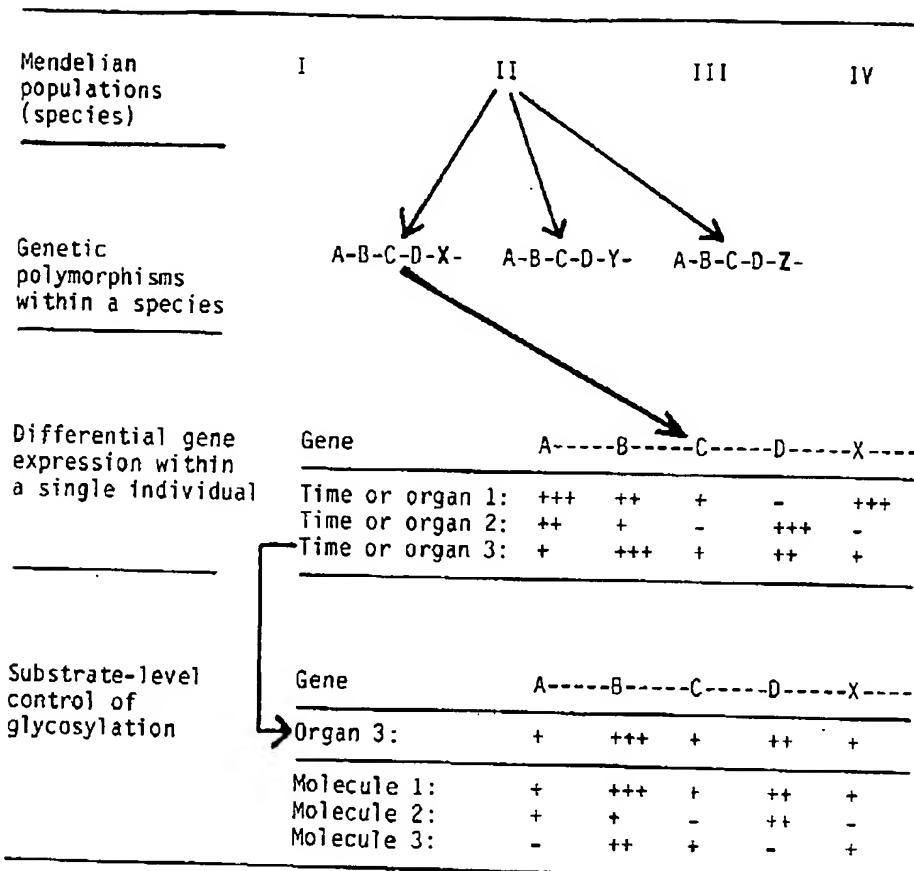


FIG. 1. Hierarchies of control involved in complex carbohydrate assembly. Species differ in the genes controlling glycosylation. Within a single species, there is genetic polymorphism (genes, X, Y, and Z in the above scheme may, for example, code for the blood group A-dependent  $\alpha$ 3-Gal-transferase, the blood group B-dependent  $\alpha$ 3-Gal-transferase, or the absence of either transferase). Differential gene expression between organs or at different times in the same organ occurs during differentiation. Even in a single organ at a particular time, substrate-level factors may modify the activity of a glycosyltransferase.

functions in higher organisms, e.g., the role of cell surface carbohydrates in the interactions of cells with their cellular and fluid environments. There is preliminary evidence that the branching patterns of cell surface carbohydrate may be important in oncogenic transformation (2-5). However, the oligosaccharides on some glycoproteins show extreme microheterogeneity: e.g., highly purified hen ovomucoid has more than 20 different oligosaccharides (6, 7). This phenomenon is common and is difficult to reconcile with the hypothesis that complex carbohydrates mediate an information-transfer role.

The highly branched nature of complex carbohydrates has important implications concerning their synthesis. Proteins and nucleic acids are linear molecules and are synthesized by a highly accurate template mechanism; DNA acts as a template for RNA and RNA acts as a template for protein. Because they are branched, complex carbohydrates cannot be made in this way. Genetic information is transferred via an indirect nontemplate

pathway. Genes code for the protein backbone of the glycoprotein, for the glycosyltransferases and glycosidases that form the oligosaccharides, for substrate and cofactor availability, and for the construction of the endomembrane assembly lines within which all complex carbohydrates are synthesized. The end result is that nucleic acid and protein assembly tends to be very accurate, while the assembly of complex carbohydrates is prone to variation and leads to the phenomenon of microheterogeneity. It is not known whether this variation is random and serves little purpose or whether microheterogeneity and the large diversity of structures have a function not yet understood.

#### Biosynthetic factors involved in complex carbohydrate microheterogeneity

Figure 1 attempts to outline the various factors which contribute to the microheterogeneity of complex carbohydrates.

Mend  
Sul  
specie  
carbo  
detec  
pared  
transj  
mice  
and h  
and I  
speci  
trans  
struc  
rides  
15).  
clear

Ger  
It  
with  
Onl  
gen  
mo

TABLE I. Tissue and species survey for Gn-T\*

Enzyme source	Gn-T activity (nmol/h per milligram)				
	Core 3 β3-Gn-T	Core 4 β6-Gn-T	Core 2 β6-Gn-T	2/4 ratio	Elongation β3-Gn-T
<b>Rat</b>					
Colon	19.7	108	135	1.3	17
Stomach	0.4	25.2	29.6	1.2	1
Submaxillary gland	0	4.1	4.4	1.1	0
Small intestine	3.0	38.6	48.6	1.3	1
Liver microsomes	1.0	<0.6	<0.6	—	<0.4
<b>Pig</b>					
Colon	20.5	13.4	51.2	3.8	14
Stomach	0.8	167	334	2.0	9
Submaxillary gland	0	0	0	—	0
<b>Dog</b>					
Submaxillary gland	0	17.8	114	6.4	0
<b>Monkey</b>					
Colon	2.4	4.7	11.9	2.5	—
Stomach	0.4	9.5	25.9	2.7	—
<b>Human</b>					
Colon	5.5	10.3	24.0	2.3	0-9.6
Serum	—	—	—	—	0
<b>Sheep</b>					
Stomach	0.6	16.9	21.3	1.3	—

\*The enzyme reactions are as follows. Core 3 β3-Gn-T (15, 16): UDP-GlcNAc + GalNAc-R → GlcNAcβ1-3GalNAc-R + UDP. Core 4 β6-Gn-T (15, 16): UDP-GlcNAc + GlcNAcβ1-3GalNAc-R → GlcNAcβ1-3(GlcNAcβ1-6)GalNAc-R + UDP. Core 2 β6-Gn-T (17, 18): UDP-GlcNAc + Galβ1-3GalNAc-R → Galβ1-3(GlcNAcβ1-6)GalNAc-R + UDP. Elongation β3-Gn-T (19, 20): UDP-GlcNAc + Galβ1-3(Rβ1-6)GalNAc-R → GlcNAcβ1-3Galβ1-3(Rβ1-6)GalNAc-R + UDP.

#### Mendelian populations (or species)

Sufficient data is now available to indicate that species may differ in the nature of their complex carbohydrates. For example, Kobata and his colleagues detected species-specific differences when they compared the carbohydrate structures present on γ-glutamyl transpeptidase purified from the kidney and liver of rats, mice, and cows (2, 4, 8-12), on fibronectin from bovine and human plasma, and on α<sub>1</sub>-acid glycoprotein from rat and human plasma (13, 14). We have observed similar species-specific differences in the activities of glycosyltransferases involved in the assembly of the core structures of the O-glycosidically linked oligosaccharides found in mucin-type glycoproteins (Table 1; Ref. 15). The functional significance of these findings is not clear.

#### Genetic polymorphism within a species

Individuals within a Mendelian population, and even within a subspecies, can differ in their genetic makeup. Only true clones obtained by asexual reproduction are genetically identical. The best example of genetic polymorphism in man is due to complex carbohydrates, i.e.,

the ABO and Lewis human blood group antigens. The genes responsible for these antigens code for a series of glycosyltransferases (A-dependent α3-GalNAc-transferase, B-dependent α3-Gal-transferase, H-dependent α2-fucosyltransferase, and Lewis-dependent α4-fucosyltransferase).

There are two major forces for the above type of genetic variability: (i) mutation and recombination due to sexual matings followed by natural selection and (ii) genetic drift due to random or adaptively neutral loss of genes in sexual matings. It is interesting that not only are many apparently adaptively neutral genetic polymorphisms preserved but, in fact, some decidedly harmful polymorphisms are also preserved, e.g., sickle cell anemia. One possible reason is the concept of balanced polymorphism due to hybrid vigour, i.e., that the heterozygote has a higher survival potential than either homozygote. In the case of sickle cell anemia, the heterozygote survives malaria better than normal homozygotes. Natural selection ensures optimum survival of the species, not of the individual.

The lesson to be learned is that the survival of certain molecules through evolution may be due to a relatively

minor and undetectable selective advantage. A large amount of ATP is required to synthesize a complex carbohydrate. This implies that the organism probably derives some advantage from evolutionary preservation of at least some of these large and complex structures.

#### *Differential gene expression within an individual*

The development of the fertilized egg into a complex organism (embryogenesis) requires organ development and differentiation. This process involves quantitative and qualitative differences in gene expression rates between different organs at any one time and in a particular organ at different times. Although the controls involved in eukaryotes are not yet established, it is clear that the environment of a cell must play a role during differentiation by influencing which genes are turned off and which are turned on.

There are several lines of evidence to suggest that complex carbohydrates on the cell surface are involved in the embryogenesis and differentiation processes. Hematopoietic tissue involves the differentiation of pluripotent stem cells into T and B lymphocytes, erythrocytes, granulocytes, platelets, etc. Marked changes have been observed in glycoprotein and glycolipid patterns during both erythropoiesis and granulocyte (myeloid cell) differentiation (21–27). For example, human foetal erythrocytes carry the blood group *i* antigen which is a linear oligosaccharide consisting of Gal $\beta$ 1-4GlcNAc $\beta$ 1-3 repeating units, whereas most adults carry on their red cells the I antigen containing the branched Gal $\beta$ 1-4GlcNAc $\beta$ 1-3(Gal $\beta$ 1-4GlcNAc $\beta$ 1-6)-Gal-structure. Narasimhan and colleagues (Ref. 28 and unpublished data) have observed that human B cell lineage lymphoma lines involved in immunoglobulin synthesis contain GlcNAc-transferase III (29), the enzyme which inserts a bisecting GlcNAc in  $\beta$ 1-4 linkage to the  $\beta$ -linked Man of the core of *N*-glycosyl oligosaccharides; resting T and B lymphocytes and T-lineage acute lymphatic leukemia cells were essentially devoid of GlcNAc-transferase III activity. Changes in complex carbohydrate structure have also been observed as embryogenesis progresses (23, 24, 30–32).

Changes in the expression of complex carbohydrate antigens have been detected during oncogenesis. These tumour-associated antigens often represent retrogenetic expression of carbohydrate synthesis to a particular stage of foetal development and are called oncofoetal antigens. It is therefore not surprising that these antigens are not tumour-specific, since they are synthesized in normal foetal tissues (23, 24).

Several studies have shown differential gene expression between different organs for complex carbohydrate synthesis. Kobata and colleagues observed consistent differences between kidney and liver in their studies on  $\gamma$ -glutamyl transpeptidase (2, 4, 8–12). The most

dramatic difference was the presence of bisecting GlcNAc residues (linked  $\beta$ 1-4 to the  $\beta$ -linked Man of *N*-glycosyl oligosaccharide cores) in the enzyme from rat, cow, and mouse kidney but the complete absence of this residue from rat and mouse liver enzyme. Rat hepatoma enzyme, however, showed the presence of bisecting GlcNAc residues. We have also observed organ-specific differences in our studies on the synthesis of mucin cores (Table 1; Ref. 15). For example, the activity of  $\beta$ 3-GlcNAc-transferase responsible for synthesis of core type 3 (GlcNAc $\beta$ 1-3GalNAc) is high in the rat, pig, monkey, and human colon but is very low in the stomach and submaxillary gland.

#### *Endomembrane factors*

Even in a particular organ at a particular time, a polypeptide core may acquire different types of oligosaccharide; i.e., the individual molecules of a so-called pure glycoprotein preparation will have identical amino acid sequences but yet will differ from one another in oligosaccharide side chains (e.g.,  $\alpha$ 1-acid glycoprotein, hen ovalbumin, hen ovomucoid, transferrin, etc). Or, a single organ may make two glycoproteins with totally different oligosaccharides: e.g., the hen oviduct (6, 7, 33) makes ovalbumin with high mannose and bisected hybrid *N*-glycosyl oligosaccharides but ovomucoid, in the same organ, ends up with truncated (primarily GlcNAc-terminal antennae) bisected highly branched complex *N*-glycosyl oligosaccharides (Fig. 2).

A relatively simple explanation for microheterogeneity is that polypeptides travel through different endomembrane assembly lines, either in the same cell or in different cells. This explanation applies both to the situation in which different oligosaccharides appear at a particular amino acid position of a particular polypeptide chain and to the situation in which a single organ makes different polypeptides with different oligosaccharides. This hypothesis requires a mechanism for sorting peptides to the different assembly lines. If all molecules made by a particular organ move through identical assembly lines, then factors operative at the endomembrane level must be responsible for microheterogeneity. The remainder of this review will deal with these endomembrane or "substrate-level" factors.

#### *The endomembrane assembly line*

A great deal of information is now available on the structures of *N*- and *O*-glycosyl oligosaccharides (14, 34, 35) and on the detailed enzymatic steps involved in their synthesis (35–40). Oligosaccharide synthesis occurs on a membranous assembly line. The polypeptide backbones of all glycoproteins are synthesized on membrane-bound polyribosomes and all carbohydrate addition occurs on the luminal side of the endomembrane system.

Fig  
residu  
name  
syste

Fi  
and  
asse  
Synt  
the  
pyro  
in th

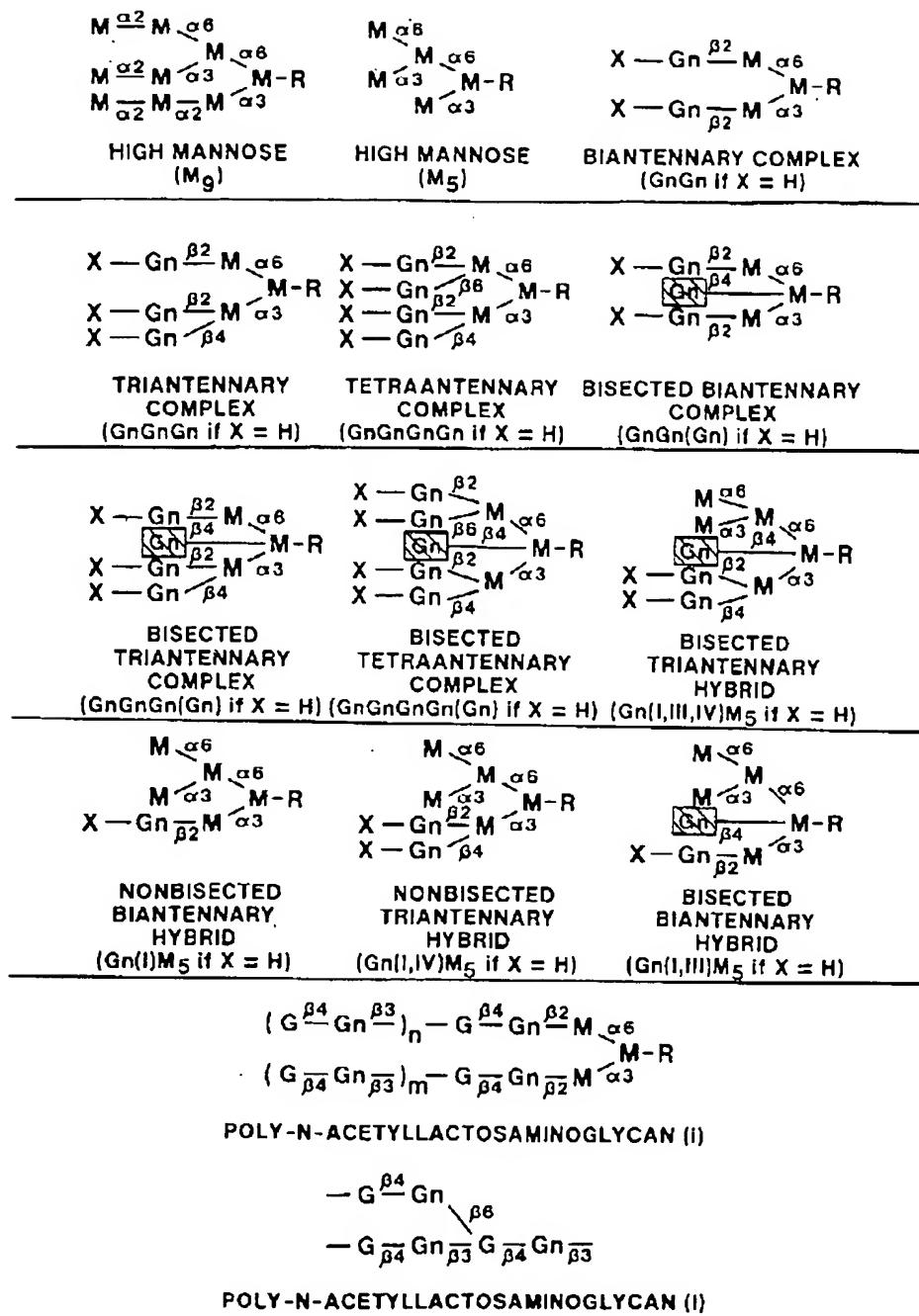


FIG. 2. Examples of *N*-glycosyl oligosaccharides (OS). High mannose OS are named according to the number of Man (M) residues, e.g., M<sub>5</sub>. Complex OS are named according to the sugars at the nonreducing termini, with the Man<sub>1-6</sub> arm being named first (M, Man; Gn, GlcNAc; G, Gal; (Gn), bisecting GlcNAc). Hybrid OS are named as shown, using the numbering system in Fig. 6 to designate the arms. X can be H, Gal<sub>1-4</sub>(3), or sialyl<sub>2-3</sub>(6)Gal<sub>1-4</sub>(3).

Figure 3, taken from the excellent review by Kornfeld and Kornfeld (35), outlines the basic steps in the assembly of biantennary *N*-glycosyl oligosaccharides. Synthesis starts in the rough endoplasmic reticulum with the transfer of a large oligosaccharide from dolichol pyrophosphate oligosaccharide to an asparagine residue in the polypeptide backbone (step 1, Fig. 3). This is

followed by oligosaccharide processing within both the rough endoplasmic reticulum (steps 2 to 4, Fig. 3) and Golgi apparatus (steps 5 and 7, Fig. 3) as the glycoprotein moves through the endomembrane assembly line.

Our laboratory has been concerned primarily with the mechanisms which determine the branching patterns of complex and hybrid *N*-glycosyl oligosaccharides (Fig.

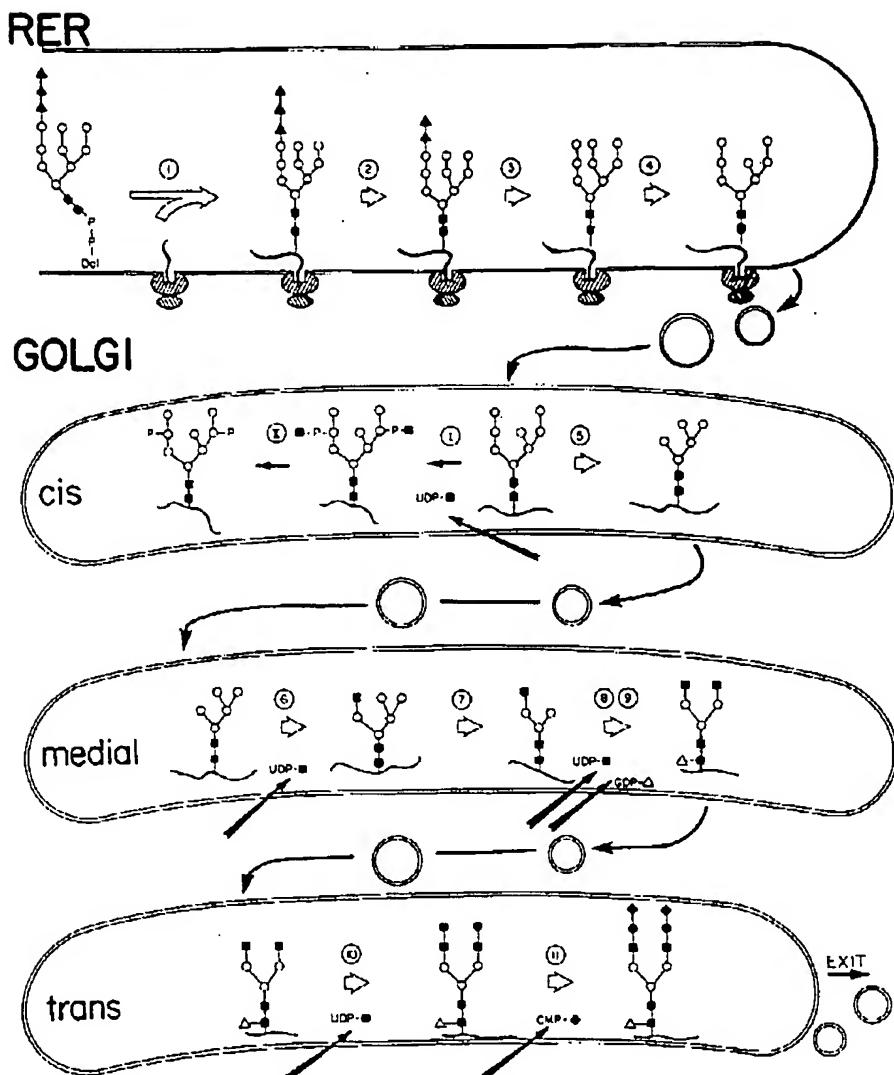


FIG. 3. Biosynthesis of *N*-glycosyl nonbisected biantennary complex oligosaccharide (from Ref. 35). Processing enzymes: (1) transfer of Glc<sub>3</sub>Man<sub>9</sub>GlcNAc<sub>2</sub> from dolichol pyrophosphate oligosaccharide to peptide nascently bound to ribosome; (2) α-glucosidase I; (3) α-glucosidase II; (4) rough endoplasmic reticulum (RER) α2-mannosidase; (5) α-mannosidase I; (6) GlcNAc-transferase I; (7) α-mannosidase II; (8) GlcNAc-transferase II; (9) α6-fucosyltransferase; (10) Gal-transferase; (11) sialyltransferase; (I) *N*-acetylglucosaminyl phosphotransferase, which acts on hydrolases destined for the lysosomes; (II) *N*-acetylglucosamine-1-phosphodiester α-*N*-acetylglucosaminidase, which exposes the Man-6-phosphate signal required for movement of hydrolases to the lysosome. Transport of nucleotide sugars into the lumen is indicated. ■, GlcNAc; ○, Man; ▲, Glc; Δ, Fuc; ●, Gal; ♦, sialic acid.

2) and with the synthesis of the four major core types of *O*-glycosyl oligosaccharides (Fig. 4). In particular, as mentioned in the previous section, we have been interested in the "substrate-level" controls which operate within the endomembrane system to produce the large variety of oligosaccharides that occur on mammalian and avian glycoproteins. These steps occur within the Golgi apparatus.

The Golgi apparatus is now believed to be subdivided into subcompartments with different functions (Fig. 3; Refs. 35, 41, and 42). The steps involved in assembly of

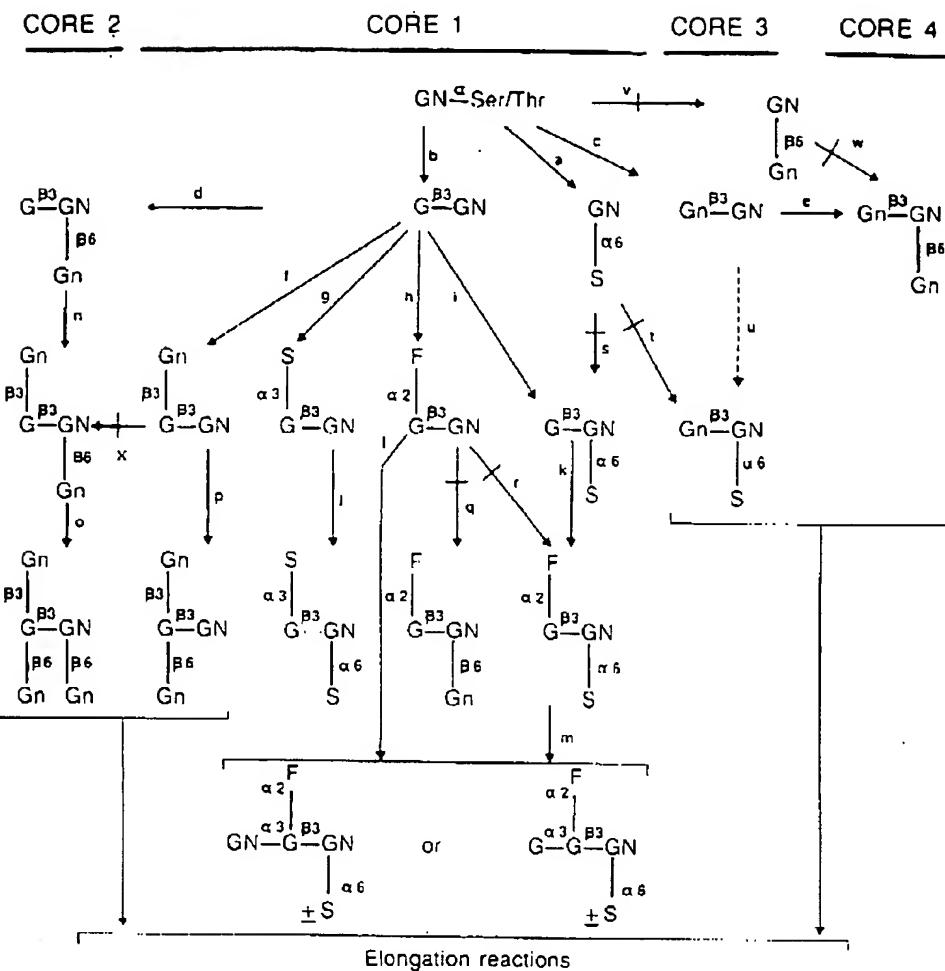
the two antennae of a typical biantennary complex *N*-glycosyl oligosaccharide probably occur in the medial and trans Golgi compartments (steps 6 and 8–11, Fig. 3). Although Fig. 3 transmits a great deal of information in a clear and concise manner, it is quite incomplete since it lacks the pathways for the synthesis of *N*-glycosyl oligosaccharides of the hybrid type and of complex types larger than biantennary. An expansion of the synthetic scheme which includes the latter structures is shown in Fig. 5. It is necessary to use a shorthand notation to fit all the pathways into a single page and the

FIG.  
Galβ  
Gn, C  
and 4

visua  
3. E  
trans

T;  
the  
subs

Con  
A  
and  
part  
enz  
are



and addition of blood group and antigenic determinants or terminal sugars

FIG. 4. Biosynthesis of *O*-glycosyl oligosaccharides. Four major core classes are shown: core 2 Gal $\beta$ 1-3GalNAc, core 1 Gal $\beta$ 1-3(GlcNAc $\beta$ 1-6)GalNAc, core 3 GlcNAc $\beta$ 1-3GalNAc, and core 4 GlcNAc $\beta$ 1-3(GlcNAc $\beta$ 1-6)GalNAc. GN, GalNAc; Gn, GlcNAc; G, Gal; S, sialic acid; F, Fuc. All steps except *u* have been proven by *in vitro* experiments (Refs. 15, 16, 19, 20, 37 and 40; I. Brockhausen, A. Koenderman, K. L. Matta, D. H. Van den Eijnden, and H. Schachter, in preparation).

visual impact is not as clear as the diagrams used in Fig. 3. Even Fig. 5 is not complete since it omits GlcNAc-transferases V, VI, and VII (Fig. 6).

#### Control of glycoprotein synthesis at the substrate level

Table 2 lists the various mechanisms which control the assembly of protein-bound oligosaccharide at the substrate level.

#### Competition for a common substrate

At many points in the synthetic schemes for both *N*- and *O*-glycosyl oligosaccharides (Figs. 4 and 5), a particular substrate can be acted on by more than one enzyme. Although the schemes shown in Figs. 4 and 5 are composite schemes based on *in vitro* assays carried

out in different tissues, it is assumed that they approximate the *in vivo* situation and that competition for a common substrate will also occur *in vivo*.

Several important "crossroads" can be pointed out (Fig. 5). Gn(I)M<sub>5</sub> can be acted on by (i) GlcNAc-transferase III to form bisected biantennary five-Man hybrids (43), (ii) GlcNAc-transferase IV to form nonbisected triantennary five-Man hybrids (43), (iii)  $\alpha$ 6-fucosyltransferase (44), or (iv)  $\alpha$ -mannosidase II to form MGn, or (v) can move from the medial to the trans Golgi to form nonbisected biantennary five-Man hybrids. Once the bisecting GlcNAc has been inserted by GlcNAc-transferase III,  $\alpha$ -mannosidase II can no longer act (45) and the pathway is fixed into the production of hybrid structures (Fig. 5). The rules permit the formation of five-Man hybrid structures with a core  $\alpha$ 6-fucose

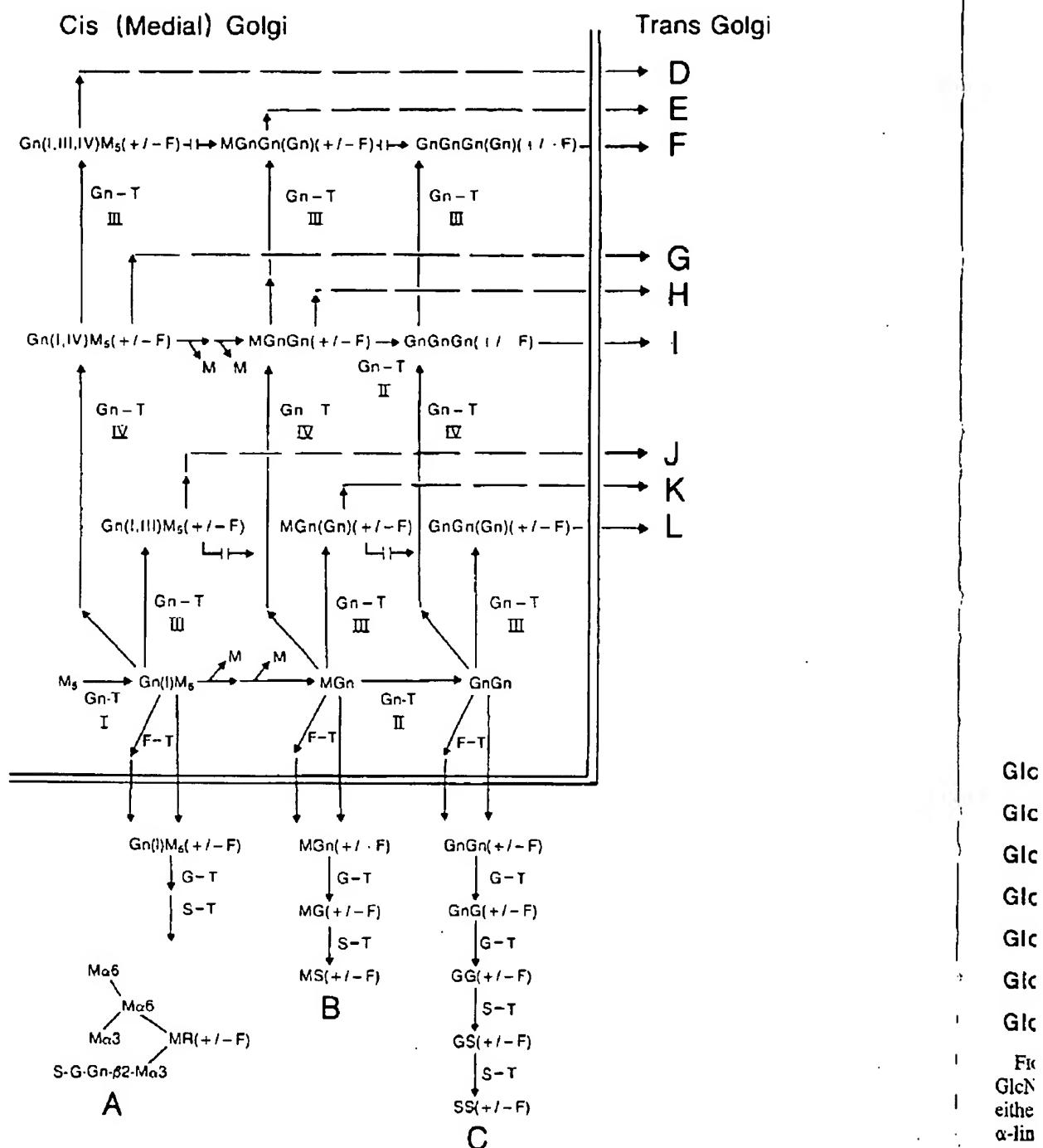


FIG. 5. Biosynthesis of *N*-glycosyl oligosaccharides (OS) showing the conversion of  $M_5$  into the following. (A) Five-Man nonbisected biantennary hybrid OS; (B) three-Man nonbisected biantennary hybrid OS (or incomplete biantennary complex OS); (C) nonbisected biantennary complex OS; (D) five-Man bisected triantennary hybrid OS; (E) three-Man bisected triantennary hybrid OS (or incomplete bisected triantennary complex OS); (F) bisected triantennary complex OS; (G) five-Man nonbisected triantennary hybrid OS; (H) three-Man nonbisected triantennary hybrid OS (or incomplete triantennary complex OS); (I) nonbisected triantennary complex; (J) five-Man bisected biantennary hybrid OS; (K) three-Man bisected biantennary hybrid OS (or incomplete bisected biantennary complex OS); (L) bisected biantennary complex. M, Man; Gn, GlcNAc; G, Gal; S, sialic acid; (Gn), bisecting GlcNAc; F, Fuc; F-T, fucosyltransferase; Gn-T, GlcNAc-transferase; S-T, sialyltransferase; G-T, Gal-transferase.

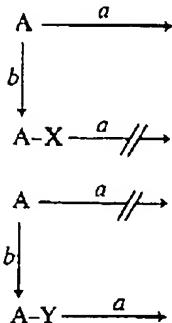
TABLE 2. Control of glycoprotein synthesis at the substrate level

## 1. Competition for a common substrate

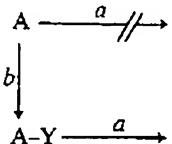


## 2. Substrate specificity of glycosyltransferases

## (i) GO - NO GO



## (ii) NO GO - GO



## (iii) Recognition site distinct from catalytic site

## (iv) Branch specificity

## (v) Role of polypeptide

## 3. Substrate availability

## (i) Subcellular compartments as assembly lines

## (ii) Transport of nucleotide sugars across membranes

## 4. Other factors: pH, cations, phosphatases, glycosidases. Phospholipid environment

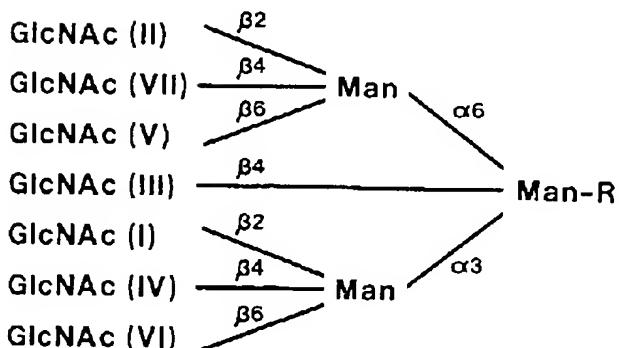


FIG. 6. A hypothetical structure showing all the antennary GlcNAc residues described to date. GlcNAc linked 1-3 to either arm of the core, 1-2 to the  $\beta$ -linked Man, or in  $\alpha$ -linkages are theoretically possible, but have not yet been described. GlcNAc residues have been numbered arbitrarily as indicated. This numbering system is used to name hybrid oligosaccharides (Fig. 2) and GlcNAc-transferases.

residue, but such structures have not as yet been reported.

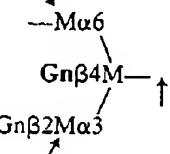
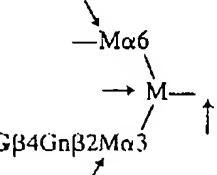
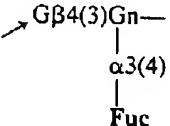
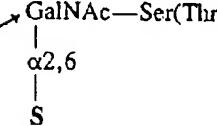
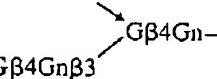
MGn can be acted on by GlcNAc-transferases II, III, or IV, or by  $\alpha$ 6-fucosyltransferase, or can enter the trans Golgi. The GlcNAc-transferase II catalyzed conversion of MGn to GnGn is probably the "main-line" pathway

(step 8, Fig. 3). GnGn is the main entry point into all complex N-glycosyl oligosaccharides. GnGn can be acted on by (i) GlcNAc-transferase III to form bisected biantennary complex structures, (ii) either GlcNAc-transferase IV or V to form nonbisected triantennary complex structures, or (iii)  $\alpha$ 6-fucosyltransferase; or (iv) can enter the trans Golgi to be acted on by  $\beta$ 4-Gal-transferase (Fig. 5).

The pathway for O-glycosyl oligosaccharide synthesis shows similar "crossroads" (Fig. 4). For example, GalNAc-Ser(Thr)-R may be converted to core type 1 (path b, Fig. 4), core type 3 (path c, Fig. 4), or sialyl $\alpha$ 2-6GalNAc-Ser(Thr)-R (path a, Fig. 4). Core type 1 (Gal $\beta$ 1-3GalNAc-R) may be acted on by several different enzymes (paths f-i, Fig. 4). Various other crossroads are evident in Figs. 4 and 5.

The existence of these competition points means that many different oligosaccharides can be made in a single assembly line depending on the relative activities of competing enzymes and may explain, at least in part, the microheterogeneity of oligosaccharides at a single amino acid site. In addition, tissues and species probably differ in their complement of competing enzymes, resulting in the differences in structure that have been observed. For example, ovine submaxillary glands have a higher ratio of CMP-sialic acid:GalNAc-R  $\alpha$ 6-sialyl-

TABLE 3. Substrate specificity: GO - NO GO\*

X	a	Ref.
	$\alpha$ -Mase II Gn-T II and IV $\alpha$ 6-Fuc-T	45 46 44
	$\alpha$ -Mase II Gn-T II, III, and IV Gn-T V† $\alpha$ 6-Fuc-T†	45 29, 46 47 44
	Blood group enzymes: A-dependent $\alpha$ 3-GalNAc-T to Gal B-dependent $\alpha$ 3-Gal-T to Gal H-dependent $\alpha$ 2-Fuc-T to Gal	48
	$\beta$ 3-Gal-T to GalNAc	49
	Blood group I-dependent $\beta$ 6-Gn-T to Gal	50

\*M, mannose; Gn, N-acetylglucosamine; G, galactose; S, sialic acid; Fuc, fucose; Gn-T, N-acetylgalactosaminyltransferase. The residue in bold print is X, the NO GO signal. The arrows indicate the site of action of the enzymes listed under a.

†These enzymes have only been tested with substrates in which there is a Gal residue on both antennae.

transferase (path *a*, Fig. 4) to UDP-Gal:GalNAc-R  $\beta$ 3-Gal-transferase (path *b*, Fig. 4) than do porcine submaxillary glands; the  $\alpha$ 6-sialyl residue prevents  $\beta$ 3-Gal-transferase action (path *s*, Fig. 4) and, therefore, ovine submaxillary mucin contains primarily sialyl $\alpha$ 2-6GalNAc chains whereas porcine submaxillary mucin contains mainly larger chains. The interesting point is that these two mucins differ quantitatively rather than qualitatively in their oligosaccharide compositions. Table 1 lists other tissue and species differences for several of the mucin glycosyltransferases.

#### Substrate specificity GO - NO GO

One of the most important directing forces in oligosaccharide biosynthesis are the constraints imposed by the specific substrate requirements of many of the glycosyltransferases. Some of these properties will now be reviewed. The first example has been named the GO - NO GO effect. This simply means that the insertion of a single critical sugar residue (X) into an oligosaccharide will convert it from a substrate to a nonsubstrate. Table 3 lists some of the most important NO GO residues.

FIG  
β1-2  
GlcN  
 $\alpha$ -f1

Tl  
actir  
Fig.  
(38,  
2Ma  
the  
(iii)  
3M:

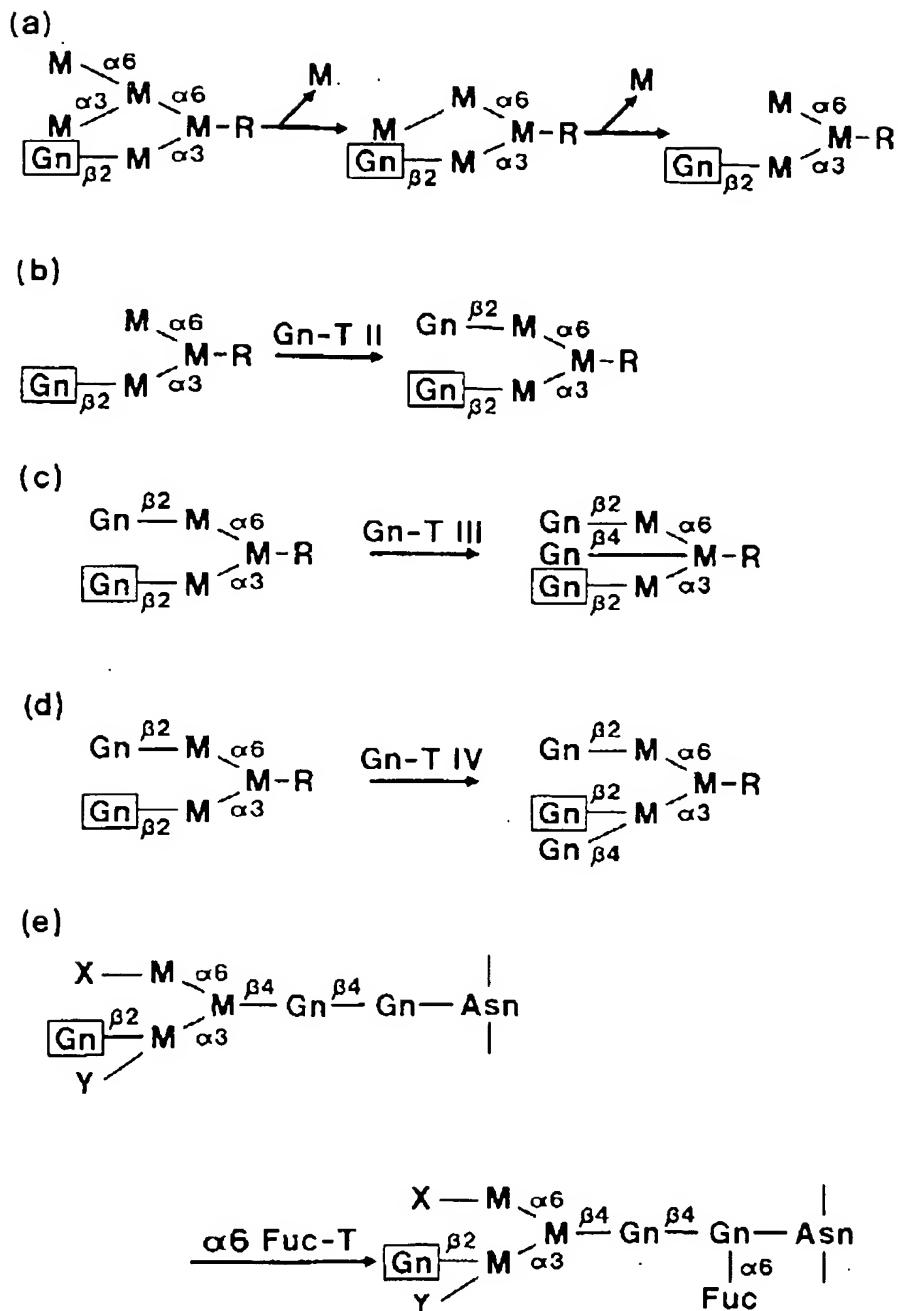


FIG. 7. Reactions catalyzed by five enzymes requiring the prior action of GlcNAc-transferase I which inserts a GlcNAc in  $\beta$ 1-2 linkage into the Man $\alpha$ 1-3 arm. (a) Mannosidase II; (b) GlcNAc-transferase II; (c) GlcNAc-transferase III; (d) GlcNAc-transferase IV; (e)  $\alpha$ 6-fucosyltransferase. M, Man; Gn, GlcNAc; Gn-T, GlcNAc transferase; Fuc, fucose;  $\alpha$ 6Fuc-T,  $\alpha$ 6-fucosyltransferase.

igo-  
I by  
the  
low  
O-  
of a  
ride 3

The first two examples refer to a group of enzymes acting on *N*-glycosyl oligosaccharides (illustrated in Fig. 7) which have in common the following properties (38, 51): (i) they require the presence of the GlcNAc $\beta$ 1-2Man $\alpha$ 1-3Man $\beta$ 1-4 grouping, (ii) activity is blocked if the substrate contains a bisecting GlcNAc (Fig. 7), and (iii) activity is blocked if the GlcNAc $\beta$ 1-2Man $\alpha$ 1-3Man $\beta$ 1-4 antenna is covered at its nonreducing end by

a Gal $\beta$ 1-4- residue. We explain these data by assuming that these enzymes all require the GlcNAc $\beta$ 1-2Man $\alpha$ 1-3Man $\beta$ 1-4 grouping as a recognition site distinct from the catalytic site and that this recognition site is blocked either by a bisecting GlcNAc or by a Gal residue. The structures shown in Figs. 8 and 9 are based on three-dimensional studies by nuclear magnetic resonance spectrometry (34) and illustrate the steric hin-

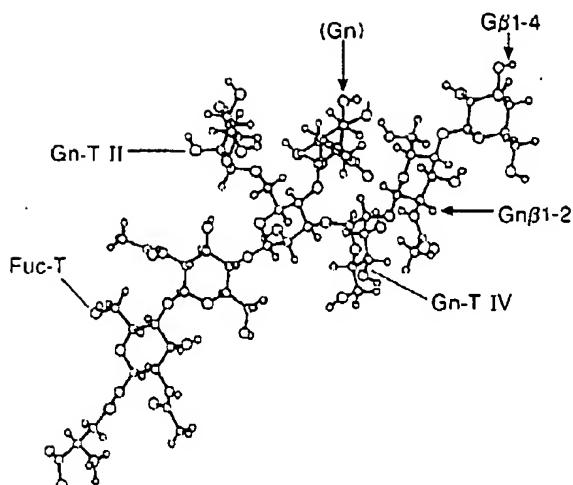


FIG. 8. Computer-drawn structure for MG(Gn),  $\omega = +180^\circ$ , based on NMR data of Carver and Brisson (34). The sites of action of GlcNAc-transferases II and IV (Gn-T II and IV) and of  $\alpha 6$ -fucosyltransferase (Fuc-T) are indicated. M, Man; Gn, GlcNAc; (Gn), bisecting GlcNAc; G, Gal.

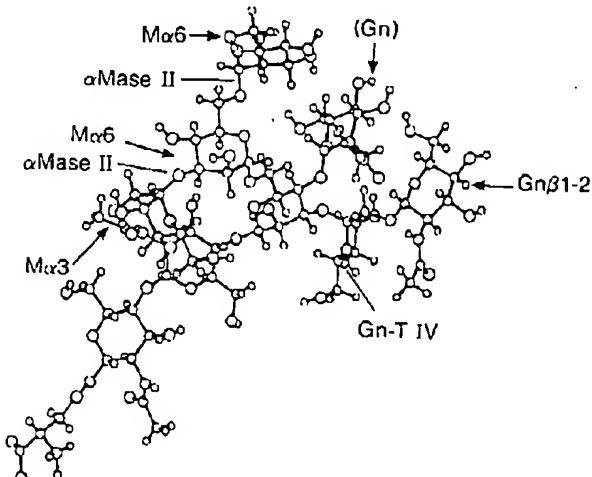


FIG. 9. Computer-drawn structure for Gn(I,III)M<sub>5</sub>, based on NMR of Carver and Brisson (34). The sites of action of  $\alpha$ -mannosidase II ( $\alpha$ -Mase II) are indicated. Abbreviations are as for Fig. 8.

drance of the recognition site by either the bisecting GlcNAc or Gal residues. Figure 8 shows that the sites of action of GlcNAc-transferases II and IV and of  $\alpha 6$ -fucosyltransferase are accessible and well removed from the sterically blocked GlcNAc $\beta 1-2$ Man $\alpha 1-3$ Man $\beta 1-4$  site; Fig. 9 shows a similar situation for  $\alpha$ -mannosidase II.

Other NO GO residues are the following. (i) A fucose linked  $\alpha 1-3$  or  $\alpha 1-4$  to a penultimate GlcNAc residue will prevent the action of blood group A-dependent  $\alpha 3$ -GalNAc-transferase, blood group B-dependent  $\alpha 3$ -Gal-transferase, and blood group H-dependent  $\alpha 2$ -fucosyltransferase. (ii) As mentioned in the previous sec-

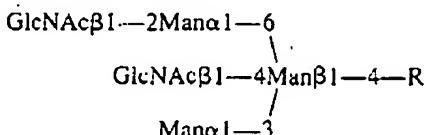
tion, a sialyl residue linked  $\alpha 2-6$  to GalNAc prevents the action of the mucin core 1  $\beta 3$ -Gal-transferase. (iii) A Gal $\beta 1-4$  residue on the GlcNAc $\beta 1-3$ Gal $\beta 1-4$ -terminus prevents the action of blood group I  $\beta 6$ -GlcNAc-transferase. These examples lend support to the concept of carbohydrate recognition sites remote from the catalytic sites.

#### NO GO - GO

The NO GO - GO effect is the reverse phenomenon: i.e., the insertion of a single critical sugar residue (Y) into an oligosaccharide will convert it from a nonsubstrate to a substrate. Two typical examples are shown in Table 4. The first example is at an important decision point in the synthesis of *N*-glycosyl oligosaccharides. If GlcNAc-transferase I does not insert a GlcNAc residue on the Man $\alpha 1-3$  arm (step 6, Fig. 3), synthesis of hybrid and complex *N*-glycosyl oligosaccharides cannot occur. This GlcNAc residue is a GO signal for all enzymes shown in Fig. 7 and provides a recognition site for them, as explained in the previous section. The second example in Table 4 has been called the "3-before-6" rule and applies to three enzymatic activities which are probably due to the action of a single  $\beta 6$ -GlcNAc-transferase (Ref. 15; I. Brockhausen and H. Schachter, unpublished data). The rule states that the  $\beta 6$ -GlcNAc-transferase(s) responsible for synthesis of mucin core 2 (path *d*, Fig. 4) and core 4 (path *e*, Fig. 4) and for the branch point of the blood group I determinant (paths *o* and *p*, Fig. 4) requires substitution of C-3 of the target sugar residue before C-6 can be substituted. Put another way, core 1 synthesis precedes core 2 synthesis, and core 3 synthesis precedes core 4 synthesis.

Since Gal residues appear to be needed for *i* and I antigenic activities, it is not strictly correct to state that blood group *i* synthesis precedes blood group I synthesis. However, the  $\beta 3$ -GlcNAc-transferase must act before the  $\beta 6$ -GlcNAc-transferase. For example, pig stomach mucosal extracts can transfer GlcNAc in  $\beta 1-3$  linkage to the terminal Gal of Gal $\beta 1-3$ (GlcNAc $\beta 1-6$ )-GalNAc-R but not to GlcNAc $\beta 1-6$ Gal $\beta 1-3$ (GlcNAc $\beta 1-6$ )-GalNAc-R (19). Further, we have detected both the GlcNAc $\beta 1-3$ Gal— and GlcNAc $\beta 1-3$ (GlcNAc $\beta 1-6$ )-Gal— structures but not the GlcNAc $\beta 1-6$ Gal structure as enzyme products (Refs. 19 and 20; I. Brockhausen and H. Schachter, unpublished data).

The above rules, like all the other rules stated in this review, may have exceptions. For example, Yamashita et al. (7) reported the presence of the following structure in hen ovomucoid:



T  
it in  
acte  
grou  
4.6'  
fort  
A  
Glc  
hav  
cha  
mu  
fron  
rela  
tran  
ana  
nat  
Glc  
pro  
 $\beta 6$   
we  
su  
frc  
ou

TABLE 4. Substrate specificity: NO GO - GO\*

Y	a	Ref.
$\begin{array}{c} \downarrow \\ \text{—M}\alpha\text{6} \\ \rightarrow \text{M} \\ \uparrow \\ \text{Gn}\beta\text{2M}\alpha\text{3} \end{array}$	$\alpha$ -Mase II Gn-T II, III, and IV Gn-T V $\alpha$ 6-Fuc-T	45 29, 46 47 44
$\begin{array}{c} \text{G}\beta\text{3GalNAc—Ser(Thr)} \\ \text{or} \\ \text{Gn}\beta\text{3GalNAc—Ser(Thr)} \end{array}$	Mucin core 2 and (or) 4 $\beta$ 6-Gn-T to GalNAc	17 18 15, 16
$\begin{array}{c} \searrow \\ \text{G}\beta\text{4R} \\ \text{Gn}\beta\text{3} \end{array}$	Blood group I-dependent $\beta$ 6-Gn-T to Gal	50, 52, †

\*M, Man; Gn, GlcNAc; G, Gal; Gn-T, GlcNAc-transferase. The residue in bold print is Y, the GO signal. The arrows indicate the site of action of the enzymes listed under a.

†I. Brockhausen, A. Koenderman, K. L. Maitta, D. H. Van den Eijnden, and H. Schachter, in preparation.

This structure does not fit the synthetic rules because it implies that both GlcNAc-transferases II and III have acted without the presence of the GlcNAc $\beta$ 1-2Man $\alpha$ 1-3-grouping. The above compound represented less than 4.6% of the total carbohydrate and may have been formed by glycosidase action.

Another exception to the rules is the report of GlcNAc $\beta$ 1-6GalNAc in human  $\kappa$ -casein (53). We have been unable to detect the synthesis of this disaccharide *in vitro* in our systems. For example, GalNAc-mucin was incubated with UDP-GlcNAc and enzyme from either pig colon or rat colon, and the product was released by alkaline borohydride and purified by gel filtration and high performance liquid chromatography; analysis by high resolution nuclear magnetic resonance spectrometry and methylation analysis revealed GlcNAc $\beta$ 1-3GalNAcOH to be the only disaccharide product (15). Perhaps human tissues have an unusual  $\beta$ 6-GlcNAc-transferase or the residue on C-3 of GalNAc was incorporated and subsequently lost. What is reassuring is that the great majority of structures isolated from many different glycoproteins fit the synthetic rules outlined above.

#### Recognition sites distinct from catalytic sites

Table 5 gives examples which support the concept of recognition sites distinct from catalytic sites. We have already discussed the first example in which GlcNAc $\beta$ 1-2Man $\alpha$ 1-3Man $\beta$ 1-4- serves as a recognition site for the enzymes listed in Fig. 7. The second example is the requirement shown by GlcNAc-transferase I for a Man $\alpha$ 1-3Man $\beta$ 1-4GlcNAc- moiety (51). The third example was reported by Joziasse et al. (54) for bovine colostrum CMP-sialic acid:Gal $\beta$ 1-4GlcNAc-R  $\alpha$ 2-6-sialyltransferase; they noted a partial requirement for an intact GlcNAc ring in the Man $\beta$ 1-4GlcNAc sequence of the N-glycosyl oligosaccharide core and postulated that the sialyltransferase required this region for optimum positioning on the substrate.

#### Branch specificity

Van den Eijnden and co-workers have coined the term branch specificity to describe the preferential action of a glycosyltransferase on a particular arm of a branched oligosaccharide (Table 6). For example, elongation of biantennary complex N-glycosyl oligosaccharides by both  $\beta$ 4-Gal-transferase and  $\alpha$ 2-6-sialyltransferase oc-

TABLE 5. Substrate specificity: recognition sites distinct from catalytic sites

Recognition site	Enzymes	Ref.
	α-Mase II GlcNAc-T II, III, and IV GlcNAc-T V α6-Fuc-T	45 29, 46 47 44
	GlcNAc-T I α2-6-Sialyl-T to Galβ4GlcNAc—	51 54

TABLE 6. Substrate specificity: branch specificity

Branched oligosaccharide	Enzyme	Preference	Ref.
R1—Mα6	β4-Gal-T	Mα3 arm	55
Mβ4—R	α6-Sialyl-T	Mα3 arm	56
R2—Mα3	α3-Gal-T	Mα6 arm	57
R1—Gnβ6	α3-Gal-T	Gnβ6 arm	58
Gal—R	β4-Gal-T	Gnβ6 arm	59
R2—Gnβ3			

curs preferentially on the  $\text{Man}\alpha 1\text{-}3$  arm. This may be due, in part, to the fact that the  $\text{Man}\alpha 1\text{-}6$  arm can exist in two states in equilibrium with each other; one of these states may not be as readily accessible as the other (Ref. 34; Figs. 10 and 11). However, the fact that  $\alpha 3$ -Gal-transferase from calf thymus prefers the  $\text{Man}\alpha 1\text{-}6$  arm (58) makes this argument somewhat suspect. The indications are that branch specificities found *in vitro* reflect structural variations that occur *in vivo*.

#### Role of polypeptide

The *in vitro* glycosyltransferase studies described above were carried out either with well-characterized low molecular weight acceptors or relatively poorly characterized glycoprotein acceptors. It is as yet not possible to obtain a pure large polypeptide with a homogeneous oligosaccharide at a single amino acid

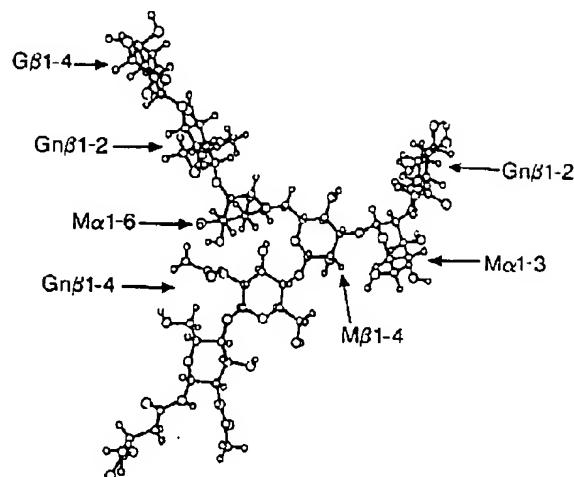


FIG. 10. Computer-drawn structure for GGn,  $\omega = -60^\circ$ , based on NMR data of Carver and Brisson (34). Abbreviations are as for Fig. 8.

position. Thus, the role of the polypeptide in glycosyltransferase action has not been properly studied. As noted above, however, the rules outlined by *in vitro* transferase studies are usually supported by the structures isolated from tissues. Nevertheless, there are strong indications that the polypeptide backbone often plays an important modifying role during oligosaccharide assembly.

For example, consider the situation for hen ovalbumin and hen ovomucoid (14). Both glycoproteins are made in the same organ and yet ovalbumin contains mainly high mannose and bisected hybrid structures,

Gf

Fig  
based  
are as  
when  
branch  
struc  
struc  
rich  
and  
diffe  
that  
asse  
expl  
with  
bum  
for  
whe  
tran  
T  
coic  
inte  
doe  
tran  
bise  
ster  
a bi  
the  
ant  
rep  
(14  
rol  
syr  
me  
not  
but  
we  
cor

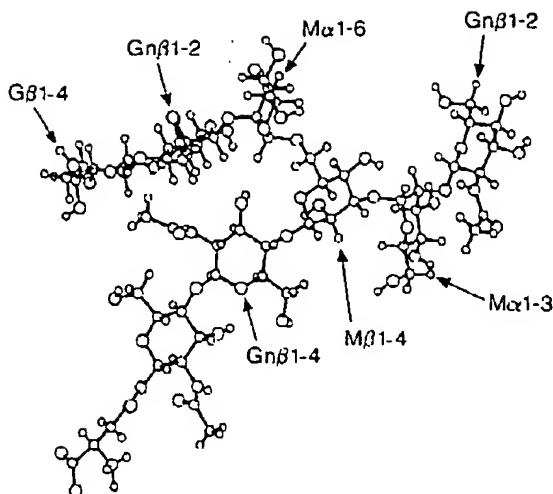


FIG. 11. Computer-drawn structure for GGn,  $\omega = +180^\circ$ , based on NMR data of Carver and Brisson (34). Abbreviations are as for Fig. 8.

whereas ovomucoid is very rich in bisected highly branched truncated (mainly GlcNAc terminal) complex structures. Both glycoproteins are rich in bisected structures and indeed we have found that hen oviduct is a rich source of GlcNAc-transferase III (29), whereas rat and pig liver lack this enzyme. But why is there a difference between the two proteins? One explanation is that the two proteins pass through separate and different assembly lines. Figure 12 suggests another possible explanation. The polypeptide backbone may interact with oligosaccharide in such a way as to make ovalbumin a good substrate and ovomucoid a poor substrate for GlcNAc-transferase III at the five-mannose stage whereas ovomucoid becomes a good substrate for the transferase at the three-mannose stage.

The reason for the undergalactosylation of ovomucoid and various other glycoproteins is not known. It is interesting that Narasimhan et al. (56) found that not only does a Gal residue on the Man $\alpha$ 1-3 arm inhibit GlcNAc-transferase III, but  $\beta$ 4-Gal-transferase is inhibited by a bisecting GlcNAc; this is perhaps not surprising on stereochemical grounds (Fig. 8). Thus prior addition of a bisecting GlcNAc may lead to undergalactosylation of the GlcNAc $\beta$ 1-2Man $\alpha$ 1-3- and GlcNAc $\beta$ 1-2Man $\alpha$ 1-6-antennae relative to the other antennae; the structures reported for ovomucoid tend to support this hypothesis (14).

Savvidou et al. (60) have provided evidence for the role of polypeptide-oligosaccharide interaction in the synthetic process. They showed that a human IgG1k monoclonal protein (Hom) had oligosaccharide moieties not only at the usual position (Asn-297 of the H chain) but also at Asn-107 on the L chain. All oligosaccharides were of the complex biantennary *N*-glycosyl type with core fucose residues and, therefore, processing at both

sites had entered the Golgi phase (Fig. 3). At this point, the H and L chains are already covalently associated and therefore both glycosylation sites must pass through the same endomembrane assembly line and encounter the same array of processing enzymes. However, the Asn-107 site was occupied entirely by mono- and di-sialyl bisected biantennary complex structures, whereas the Asn-297 site had mainly (73%) nonbisected sialylated biantennary structures and 27% bisected neutral biantennary structures. Since galactosylation and sialylation occur after the action of GlcNAc-transferase III (Fig. 5), it cannot be argued that the oligosaccharide at Asn-297 is buried in the protein and therefore not accessible to GlcNAc-transferase III. The clone of cells making this IgG was obviously rich in GlcNAc-transferase III, since the Asn-107 site is fully bisected. Why is the Asn-297 site so poorly bisected?

The explanation hinges on the assumption that GlcNAc-transferase III cannot act on the substrate conformation in which the torsional angle  $\omega$  about the C-5-C-6 bond of the Man $\alpha$ 1-6 linkage equals  $-60^\circ$  (the two antennae form a Y, Fig. 10). The substrate for GlcNAc-transferase III is the conformation in which the angle  $\omega$  is  $+180^\circ$  (the Man $\alpha$ 1-6 arm is bent backwards towards the core, Fig. 11). The basis for this assumption (61) is that, whereas the nonbisected biantennary oligosaccharide exists in an equilibrium between two thermodynamically favourable conformations ( $\omega = -60$  or  $+180^\circ$ , Figs. 10 and 11), the bisected biantennary structure is found exclusively in the orientation corresponding to  $\omega = +180^\circ$  (Fig. 8). It was suggested (61) that the oligosaccharide at Asn-107 exists in equilibrium between both conformations and is therefore accessible to GlcNAc-transferase III, whereas most of the oligosaccharide at Asn-297 is stabilized by interaction with the polypeptide in the  $\omega = -60^\circ$  conformation and cannot serve as a substrate for GlcNAc-transferase III. The reason for the 27% bisected structures at Asn-297 is not clear. Figure 13 illustrates the hypothesis. In support of this theory, elucidation of the three-dimensional structure of the F<sub>c</sub> fragment from a human immunoglobulin, determined by X-ray diffraction studies (62), showed that the nonbisected biantennary complex oligosaccharide at Asn-297 was indeed stabilized in the form with  $\omega = -60^\circ$  through interactions between the protein and the terminal Gal residue of the Man $\alpha$ 1-6 arm.

#### *Substrate availability and other factors*

It has already been pointed out that the endomembrane system is highly compartmentalized (Figs. 3 and 5). The analogy to an assembly line is obvious. The transferases and glycosidases which shape the oligosaccharide structure are arranged along the endomembrane in the order in which they are needed. This arrangement minimizes the need for the substrate to seek its proper

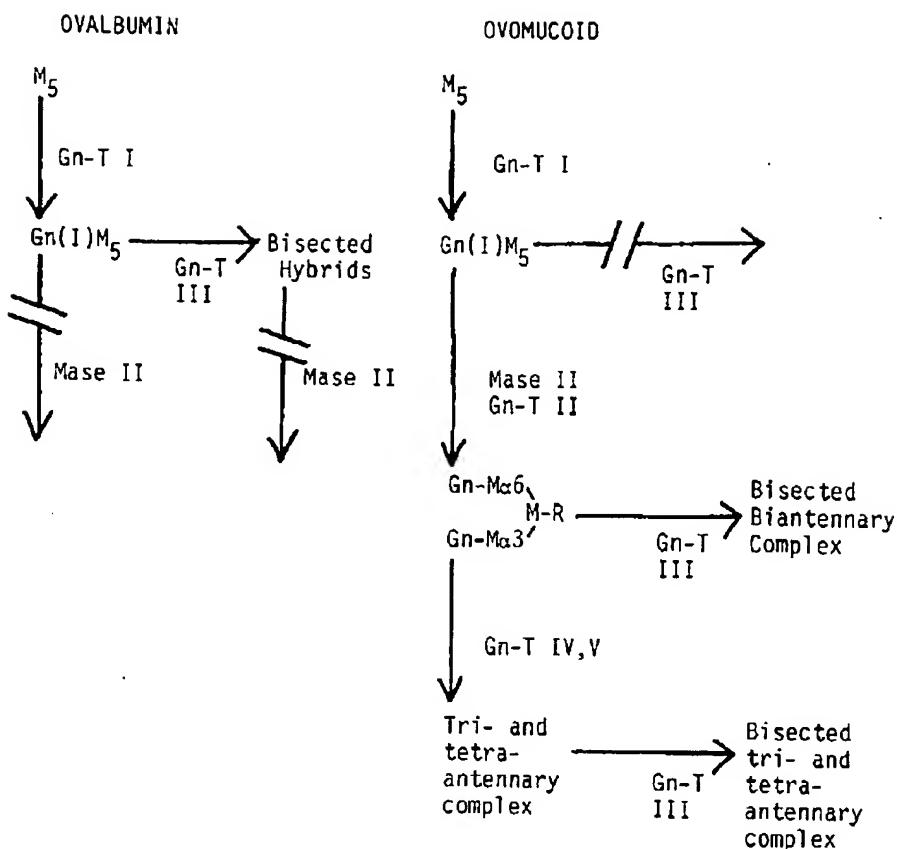


FIG. 12. Suggested mechanism for the difference between ovalbumin and ovomucoid (see text). M, Man; Gn, GlcNAc; Mase II,  $\alpha$ -mannosidase II; Gn-T, GlcNAc-transferase.

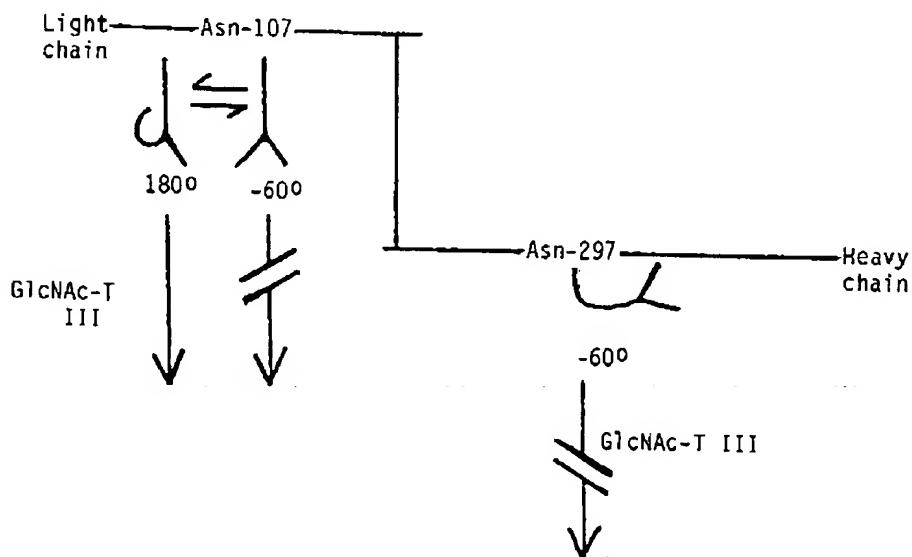


FIG. 13. A monoclonal IgG, studied by Savvidou et al. (60), was found to have bisected oligosaccharides at Asn-107 of the light chains, but mainly nonbisected oligosaccharides at Asn-297 of the heavy chains. Interaction of the oligosaccharide at Asn-297 with the protein backbone is believed to be the basis of this effect (see text).

enzyme and also exercises a certain amount of control over the assembly process.

For example, the fact that the GlcNAc-transferases which control branching (Fig. 6) are in the medial Golgi compartment, whereas the terminal glycosyltransferases (Gal- and sialyl-transferases) are in the trans Golgi (Fig. 5), means that branching is determined before the antennae are completed. This control must be important since the cell uses yet another mechanism to ensure that it is carried out. Once the glycoprotein enters the trans Golgi, it is galactosylated (Fig. 5) and this immediately prevents further action of GlcNAc-transferases II, III, IV, and V and of  $\alpha$ -fucosyltransferase. Thus, even if there are small amounts of these medial Golgi enzymes in the trans Golgi, they are quickly prevented from acting. The reason for this stringent control is not known.

Several laboratories have reported transport proteins within the membranes of the endomembrane system which allow the movement of nucleotide sugars from the cytoplasm into the luminal spaces. These transport mechanisms are indicated in Fig. 3 and obviously play a major role in controlling glycoprotein synthesis. Defects in these transport proteins have been shown to lead to the disruption of glycoprotein and glycolipid synthesis. However, a discussion of this important topic is beyond the scope of this review.

Finally, a variety of other substrate-level factors may exert control on the synthetic process. (i) The pH of the luminal compartment may affect transferase activity. (ii) The availability of cations is usually needed for transferase action. (iii) The presence of pyrophosphatases in the Golgi allows conversion of the nucleotide diphosphate formed after glycosyltransferase action into nucleotide monophosphate. The nucleotide monophosphate is exported out of the Golgi apparatus in a counter-transport system with nucleotide sugars. (iv) Several laboratories have reported on the importance of phospholipids in glycosyltransferase activity. (v) Post-translational modifications of glycosyltransferases, e.g., phosphorylation, may play a role in modifying their activity. These factors will not be discussed in this review.

### Conclusions

It is apparent that many different factors enter into the construction of a complex carbohydrate. It is, therefore, not surprising that a large variety of structures is synthesized by the cell. We are beginning to understand some of the biosynthetic machinery involved. However, it has not been ruled out that much of the microheterogeneity of these structures is indeed a random process with little functional significance. The arguments presented in this review indicate that a great deal of control

is exerted and raise the hope that as we understand the process better the functional aspects will become more evident.

What is needed are new approaches to the problem. Figure 1 emphasizes the genetic aspects and suggests that one approach must be to obtain genetic probes for the enzyme machinery involved in biosynthesis. Indeed, several laboratories are engaged in this pursuit and a few initial successes have been reported. Perhaps within the next few years, a battery of genetic probes will become available that will allow the fine dissection of the biosynthetic pathways outlined in Figs. 4 and 5.

Another area of future research must be the three-dimensional aspects of protein-oligosaccharide interaction, particularly the role of branching. Such studies are important not only for model systems such as lectin-oligosaccharide interactions (63), but also for the proper understanding of how the biosynthetic machinery operates, of how complex carbohydrates at the cell surface control the interactions of a cell with its environment, and in fact, of all functional aspects of complex carbohydrates.

In conclusion, although tremendous advances have been made in recent years in the structure and biosynthesis of complex carbohydrates, we are only beginning to attack the problem of function.

### Acknowledgements

This research was supported by grants from the Medical Research Council of Canada and the National Institutes of Health, U.S.A. Thanks are given to D. Cumming for the preparation of Figs. 8-11.

1. Olden, K., Bernard, B. A., Humphries, M. J., Yeo, T.-K., Yeo, K.-T., White, S. L., Newton, S. A., Bauer, H. C. & Parent, J. B. (1985) *Trends Biochem. Sci.* **10**, 78-82
2. Yamashita, K., Hitoi, A., Taniguchi, N., Yokosawa, N., Tsukada, Y. & Kobata, A. (1983) *Cancer Res.* **43**, 5059-5063
3. Yamashita, K., Tachibana, Y., Ohkura, T. & Kobata, A. (1985) *J. Biol. Chem.* **260**, 3963-3969
4. Kobata, A. & Yamashita, K. (1984) *Pure Appl. Chem.* **56**, 821-832
5. Hitoi, A., Yamashita, K., Ohkura, J. & Kobata, A. (1984) *Gann* **75**, 301-304
6. Yamashita, K., Kamerling, J. P. & Kobata, A. (1982) *J. Biol. Chem.* **257**, 12809-12814
7. Yamashita, K., Kamerling, J. P. & Kobata, A. (1983) *J. Biol. Chem.* **258**, 3099-3106
8. Yamashita, K., Hitoi, A., Matsuda, Y., Tsuji, A., Katunuma, N. & Kobata, A. (1983) *J. Biol. Chem.* **258**, 1098-1107
9. Yamashita, K., Hitoi, A., Tateishi, N., Higashi, T., Sakamoto, Y. & Kobata, A. (1983) *Arch. Biochem. Biophys.* **225**, 993-996

10. Yamashita, K., Tachibana, Y., Hitoi, A., Matsuda, Y., Tsuji, A., Katunuma, N. & Kobata, A. (1983) *Arch. Biochem. Biophys.* 227, 225-232
11. Yamashita, K., Tachibana, Y., Shichi, H. & Kobata, A. (1983) *J. Biochem. (Tokyo)* 93, 135-147
12. Yamashita, K., Hitoi, A., Tachibana, Y., Higashi, T., Sakamoto, Y., & Kobata, A. (1985) *Arch. Biochem. Biophys.* 240, 573-582
13. Kobata, A. (1982) in *Structure, Dynamics and Biogenesis of Biomembranes* (Sato, R. & Ohnishi, S., eds.), pp. 97-112, Japan Scientific Society Press, Tokyo
14. Kobata, A. (1984) in *Biology of Carbohydrates* (Ginsburg, V. & Robbins, P. W., eds.), vol. 2, pp. 87-161, John Wiley & Sons, New York
15. Brockhausen, I., Matta, K. L., Orr, J. & Schachter, H. (1985) *Biochemistry* 24, 1866-1874
16. Brockhausen, I., Rachaman, E. S., Matta, K. L. & Schachter, H. (1983) *Carbohydr. Res.* 120, 3-16
17. Williams, D. & Schachter, H. (1980) *J. Biol. Chem.* 255, 11247 - 11252
18. Williams, D., Longmore, G., Matta, K. L. & Schachter, H. (1980) *J. Biol. Chem.* 255, 11253 - 11261
19. Brockhausen, I., Williams, D., Matta, K. L., Orr, J. & Schachter, H. (1983) *Can. J. Biochem. Cell Biol.* 61, 1322-1333
20. Brockhausen, I., Orr, J. & Schachter, H. (1984) *Can. J. Biochem. Cell Biol.* 62, 1081-1090
21. Fukuda, M. (1985) *Biochim. Biophys. Acta* 780, 119-150
22. Fukuda, M. & Fukuda, M. N. (1984) in *The Biology of Glycoproteins* (Ivatt, R. J., ed.), pp. 183-234, Plenum Press, New York
23. Feizi, T. (1984) *Biochem. Soc. Trans.* 12, 545-549
24. Hakomori, S.-I. (1985) *Cancer Res.* 45, 2405-2414
25. Hakomori, S.-I., Fukuda, M. & Nudelman, E. (1982) in *Teratocarcinoma and Embryonic Cell Interactions* (Muramatsu, T., Gachelin, G., Moscona, A. A. & Ikawa, Y., eds.), pp. 179-200, Japan Scientific Society Press, Tokyo
26. Kannagi, R., Levery, S. B. & Hakomori, S.-I. (1983) *Proc. Natl. Acad. Sci. U.S.A.* 80, 2844-2848
27. Testa, U., Henri, A., Bettai, A., Titeux, M., Vainchenker, W., Tonthat, H., Docklear, M. C. & Rochant, H. (1982) *Cancer Res.* 42, 4694-4700
28. Narasimhan, S., Shirley, M., Hewitt, J. M., Freedman, M. H., Gelfand, E. W. & Schachter, H. (1983) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* 42, 2199
29. Narasimhan, S. (1982) *J. Biol. Chem.* 257, 10235 - 10242
30. Fenderson, B. A., Zehavi, U. & Hakomori, S.-I. (1984) *J. Exp. Med.* 160, 1591-1596
31. Gooi, H. C., Feizi, T., Kapadia, A., Knowles, B. B., Solter, D. & Evans, J. M. (1981) *Nature (London)* 292, 156-158
32. Ivatt, R. J. (1984) in *The Biology of Glycoproteins* (Ivatt, R. J., ed.), pp. 95-181, Plenum Press, New York
33. Yamashita, K., Tachibana, Y., Hitoi, A. & Kobata, A. (1984) *Carbohydr. Res.* 130, 271-288
34. Carver, J. P. & Brisson, J.-R. (1984) in *Biology of Carbohydrates* (Ginsburg, V. & Robbins, P. W., eds.), vol. 2, pp. 289-331, John Wiley & Sons, New York
35. Kornfeld, R. & Kornfeld, S. (1985) *Annu. Rev. Biochem.* 54, 631-664
36. Snider, M. D. (1984) in *Biology of Carbohydrates* (Ginsburg, V. & Robbins, P. W., eds.), vol. 2, pp. 163-198, John Wiley & Sons, New York
37. Sadler, J. E. (1984) in *Biology of Carbohydrates* (Ginsburg, V. & Robbins, P. W., eds.), vol. 2, pp. 199-288, John Wiley & Sons, New York
38. Schachter, H., Narasimhan, S., Gleeson, P. & Vella, G. J. (1983) *Can. J. Biochem. Cell Biol.* 61, 1049-1066
39. Schachter, H., Narasimhan, S., Gleeson, P., Vella, G. & Brockhausen, I. (1985) in *The Enzymes of Biological Membranes* (Martonosi, A. N., cd.), 2nd ed., vol. 2, pp. 227-277, Plenum Press, New York
40. Schachter, H. & Williams, D. (1982) *Adv. Exp. Med. Biol.* 144, 3-28
41. Rothman, J. E. (1985) *Sci. Am.* 253, 74-89
42. Dunphy, W. G. & Rothman, J. E. (1985) *Cell* 42, 13-21
43. Allen, S. D., Tsai, D. & Schachter, H. (1984) *J. Biol. Chem.* 259, 6984-6990
44. Longmore, G. D. & Schachter, H. (1982) *Carbohydr. Res.* 100, 365-392
45. Harpaz, H. & Schachter, H. (1980) *J. Biol. Chem.* 255, 4894-4902
46. Gleeson, P. A. & Schachter, H. (1983) *J. Biol. Chem.* 258, 6161-6173
47. Cummings, R. D., Trowbridge, I. S. & Kornfeld, S. (1982) *J. Biol. Chem.* 257, 13421 - 13427
48. Beyer, T. A., Sadler, J. E., Rearick, J. I., Paulson, J. C. & Hill, R. L. (1981) *Adv. Enzymol. Relat. Areas Mol. Biol.* 52, 23-175
49. Schachter, H., McGuire, E. J. & Roszman, S. (1971) *J. Biol. Chem.* 246, 5321-5328
50. Piller, F., Cartron, J.-P., Maranduba, A., Veyrieres, A., Lcroy, Y. & Fournet, B. (1984) *J. Biol. Chem.* 259, 13385 - 13390
51. Vella, G. J., Paulsen, H. & Schachter, H. (1984) *Can. J. Biochem. Cell Biol.* 62, 409-417
52. Zielenski, J. & Koscielak, J. (1983) *FEBS Lett.* 163, 114-118
53. Fiat, A. M., Jolles, P., Vliegenthart, J. F. G. & Van Halbeek, H. (1984) in *Carbohydrates, 1984, Abstracts of the XIIth International Carbohydrate Symposium* (Vliegenthart, J. F. G., Kamerling, J. P., & Veldink, G. A., eds.), p. 426, Vink Publishers, Utrecht
54. Joziasse, D. H., Schiphorst, W. E. C. M., Van den Eijnden, D. H., Van Kuik, J. A., Van Halbeek, H. & Vliegenthart, J. F. G. (1984) in *Carbohydrates, 1984, Abstracts of the XIIth International Carbohydrate Symposium* (Vliegenthart, J. F. G., Kamerling, J. P. & Veldink, G. A., eds.), p. 228, Vink Publishers, Utrecht
55. Paquet, M. R., Narasimhan, S., Schachter, H. & Moscarollo, M. A. (1984) *J. Biol. Chem.* 259, 4716-4721
56. Narasimhan, S., Freed, J. C. & Schachter, H. (1985) *Biochemistry* 24, 1694-1700
57. Van den Eijnden, D. H., Joziasse, D. H., Dorland, L.,

V:  
(!)  
58. Bl:  
(!)  
Im:  
F:  
Vi:  
59. Bl:  
Ei:

- eds.),  
ork  
chem.  
drates  
, pp.  
Gins-  
-288,
- la, G.  
66  
G. &  
ogical  
, pp.
- Med.
- 3-21  
Biol.
- hydr.
- 255,
- 'hem.
- 1, S.
- J. C.  
Mol.
- 1) J.
- , A.,  
259,
- n. J.
- 163,
- Van  
'acts  
stium  
, G.
- den  
l. &  
784,  
ypo-  
ink,
- sca-
- '85)
- L.,
- Van Halbeek, H., Vliegenthart, J. F. G. & Schmid, K. (1980) *Biochem. Biophys. Res. Commun.* 92, 839-845
58. Blanken, W. M., Van Vliet, A. & Van den Eijnden, D. H. (1984) in *Carbohydrates, 1984, Abstracts of the XIIth International Carbohydrate Symposium* (Vliegenthart, J. F. G., Kamerling, J. P. & Veldink, G. A., eds.), p. 229, Vonk Publishers, Utrecht
59. Blanken, W. M., Hoogwinkel, G. J. M. & Van den Eijnden, D. H. (1982) *Eur. J. Biochem.* 127, 547-552
60. Savvidou, G., Klein, M., Grey, A. A., Dorrington, K. J. & Carver, J. P. (1984) *Biochemistry* 23, 3736-3740
61. Brisson, J.-R. & Carver, J. P. (1983) *Can. J. Biochem. Cell Biol.* 61, 1067-1078
62. Deisenhofer, J. (1981) *Biochemistry* 20, 2361-2370
63. Carver, J. P., Mackenzie, A. E. & Hardman, K. D. (1985) *Biopolymers* 24, 49-63

ne classe  
427

National Research  
Council Canada

Conseil national  
de recherches Canada

Canada

# Biochemistry and Cell Biology

(Formerly Canadian Journal of Biochemistry  
and Cell Biology)

59.00  
92.00  
105.00

Volume 64, Number 3, March 1986

78.00  
65.00  
78.00  
63.00  
84.00

54.00  
54.00  
54.00

43.00  
55.00

clusive  
, s'ils

ents,  
1 doit  
cellu-  
(nt.),  
lés à  
nseil

ents  
sse)  
nseil

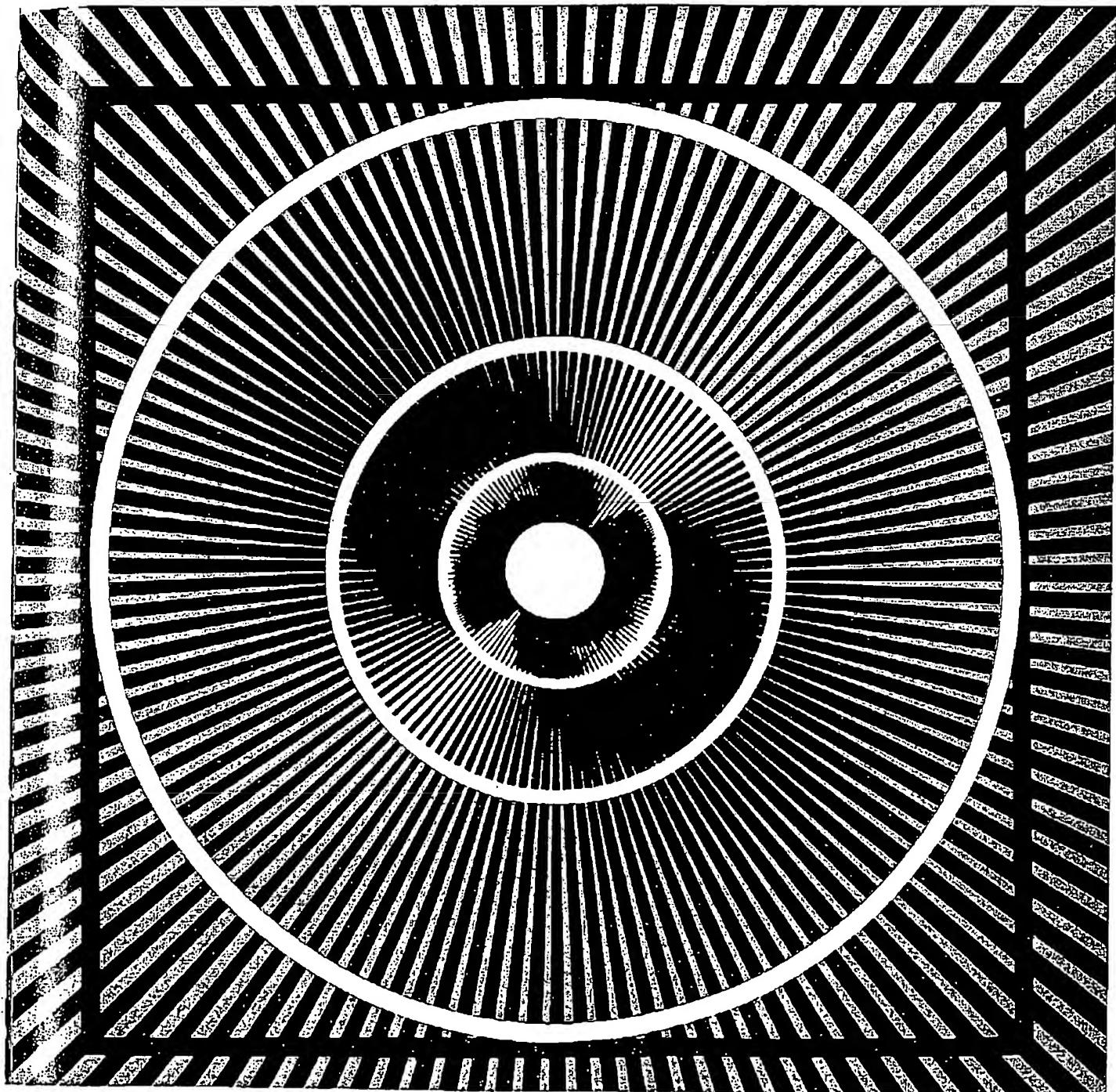
ires-  
les-  
tion.  
ules-  
ses

éro:

# Biochimie et biologie cellulaire

(Auparavant Revue canadienne de biochimie  
et biologie cellulaire)

Volume 64, numéro 3, mars 1986



# Biochemistry and Cell Biology

Published by  
THE NATIONAL RESEARCH COUNCIL OF CANADA

# Biochimie et biologie cellulaire

Publié par  
LE CONSEIL NATIONAL DE RECHERCHES DU CANADA

Editors	K. G. SCRIMGEOUR ARTHUR M. ZIMMERMAN	Directeurs scientifiques
Associate Editors/Directeurs scientifiques associés		
R. A. ANWAR	I. L. CAMERON	K. M. W. KEOUGH
B. G. ATKINSON	M. CHIRÉTIEN	W. C. McMURRAY
N. AUERSPERG	K. G. DEELEY	G. M. PADILLA
R. BLOSTEIN	A. FORER	R. H. PAINTER
P. D. BRAGG	L. GREENBAUM	H. SCHACHTER
L. BRAKIER-GINGRAS	J. W. GURD	M. S. SOLOFF
D. L. BROWN	J. M. INGRAM	M. W. SPENCE
G. S. STEIN B. D. SYKES R. M. TANGUAY J. P. WILLIAMS D. G. WILLIAMSON S. ZIMMERMAN		

PUBLISHING DEPARTMENT      SERVICE DE PUBLICATION  
at the National Research Council of Canada      du Conseil national de recherches du Canada

Editor-in-chief	C. T. BISHOP	Directeur général
Assistant Editor-in-chief	P. A. REDHEAD	Directeur général adjoint
Manager	B. E. DREW	Directrice administrative
Assistant Manager	S. D. PRUDHOMME	Directeur administratif adjoint
Publishing Supervisor	J. E. HILL	Chef des publications
Publication Officer	C. M. PEARSON	Agente de publication

Manuscripts for publication should be submitted to the appropriate Associate Editor or to the Editorial Office as indicated on pages iii and iv.

Correspondence concerning accepted manuscripts and corrected proofs should be sent to Research Journals, National Research Council of Canada, Ottawa, Ontario, Canada K1A 0R6. Telephone enquiries should be directed to J. E. Hill (613-993-0362).

Reprints may be purchased at the time of publication (an order form is sent out with the galley proof). All orders should be sent to NRCC Research Journals Desk, Printing Division, University of Toronto Press, 5201 Dufferin Street, Downsview, Ontario, Canada M3H 5T8. Invoices relating to the reprints will be sent out by the Printer.

Instructions to Authors are published once a year, in the first issue of each volume. Copies may be obtained from the Publishing Department of the journal.

The publisher grants permission to individuals who wish to quote short excerpts and reproduce figures, tables, or photographs from articles in this journal, provided that the source of such material is fully acknowledged. As a courtesy the consent of authors of such material should be obtained.

© National Research Council of Canada 1986. World rights reserved.

Authorization to photocopy items for internal or personal use, or the internal or personal use of specific clients, is granted by the National Research Council of Canada for libraries and other users registered with the Copyright Clearance Center (CCC) Transactional Reporting Service, provided that the base fee of \$3.00 per copy is paid directly to CCC, 21 Congress Street, Salem, Massachusetts, U.S.A. 01970. 0829-8211/86 \$3.00 + 0.00.

The above permission does not extend to other kinds of copying, such as copying for general distribution, for advertising or promotional purposes, for creating new collective works, or for resale. For such copying, arrangements must be made with the publisher.

Les auteurs doivent adresser leurs manuscrits aux directeurs scientifiques associés ou au bureau de rédaction tel qu'indiqué en pages iii et iv.

La correspondance ayant trait aux manuscrits acceptés et les épreuves corrigées doivent être envoyées aux Journaux de la recherche scientifique, Conseil national de recherches du Canada, Ottawa (Ontario), Canada K1A 0R6. On peut obtenir des renseignements en téléphonant à J. E. Hill (613-993-0362).

Les tirés à part peuvent être achetés au moment de la publication. Un bon de commande à cet effet accompagne les épreuves; il doit être retourné en même temps que celles-ci, non pas au bureau de la rédaction, mais à NRCC Research Journals Desk, Printing Division, University of Toronto Press, 5201 rue Dufferin, Downsview (Ontario), Canada M3H 5T8, qui se chargera d'établir la facture.

Les recommandations aux auteurs sont publiées une fois par année dans le premier numéro de chaque volume. On peut s'en procurer une copie au Service de publication.

L'éditeur accorde aux particuliers la permission de citer de brefs extraits et de reproduire des figures, des tableaux ou des photographies tirés d'articles de la revue, à la condition que la source soit indiquée explicitement. Dans ce cas, il convient, par courtoisie, d'obtenir le consentement des auteurs.

© Conseil national de recherches du Canada 1986. Tous droits réservés.

Le Conseil national de recherches du Canada accorde l'autorisation de photocopier des documents pour un usage interne ou personnel, ou pour l'utilisation interne ou personnelle de clients particuliers, aux bibliothèques et autres utilisateurs inscrits au Copyright Clearance Center (CCC) Transactional Reporting Service, à la condition que le tarif de base de 3 \$ par copie soit payé directement au Centre, au 21 Congress Street, Salem, Massachusetts, U.S.A. 01970. 0829-8211/86 3.00 + 0.00.

La présente permission ne s'applique pas à d'autres genres de reproduction, notamment la reproduction en vue d'une distribution générale, à des fins de publicité ou de promotion, pour la création de nouveaux travaux collectifs, ou pour la revente. Dans ces cas, il faut prendre les dispositions qui s'imposent en communiquant avec l'éditeur.

This journal is indexed or abstracted in / Cette revue est signalée ou résumée dans  
Biological Abstracts; Chemical Abstracts; Current Contents; Index Medicus; International Abstracts of Biological Sciences;  
IRL Life Sciences; Science Citation Index

This material may be protected by Copyright law (Title 17 U.S. Code)

Capra JD (ed): Antibody Engineering.  
Chem Immunol. Basel, Karger, 1997, vol 65, pp 111–128

## Glycosylation of Antibody Molecules: Structural and Functional Significance

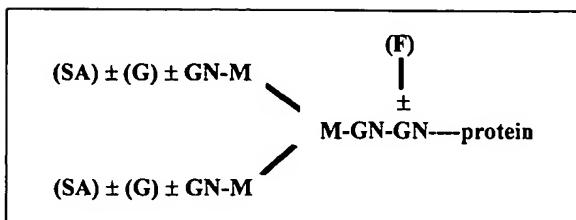
Roy Jefferis, John Lund

Department of Immunology, The Medical School, University of Birmingham,  
Birmingham, UK

The IgG antibody molecule is a structural paradigm for members of the immunoglobulin superfamily. A majority of these molecules are glycoproteins and collectively they account for ~70% of molecules currently undergoing development for possible *in vivo* therapeutic application. Whilst the oligosaccharide moiety of the IgG molecule accounts for only 2–3% of its mass, it has been shown to be essential for optimal activation of effector mechanisms leading to the clearance and destruction of pathogens. This suggests that glycosylation fidelity is an essential requirement of the IgG molecule and that it may be so for other recombinant glycoproteins produced by *in vivo* or *in vitro* techniques. Numerous studies have shown that whilst the defining biological activity of a glycoprotein molecule may not be dependent on its glycosylation, other essential characteristics are altered in aglycosylated forms, e.g. stability, pharmacokinetics, antigenicity [1–3].

The IgG molecule is composed of three globular protein moieties, two Fabs and an Fc, that are linked through a flexible ‘hinge’ region that allows freedom for multiple spatial orientations of the globular moieties with respect to each other. A flexible upper hinge region provides mobility for the Fab regions and allows the paratope of each to bind its complementary epitope. A flexible lower hinge region similarly allows Fc mobility and accessibility within antigen/antibody complexes to engage one of a variety of effector activating ligands, e.g. Fc $\gamma$  receptors, the C1 component of complement. A core hinge section is rich in proline and cystine residues, that form inter-heavy chain disulphide bridges, and has a rigid secondary structure.

Studies attempting to correlate physicochemical parameters with function were interpreted to suggest that the segmental flexibility of the hinge region



*Fig. 1.* The core carbohydrate moiety of the complex form of oligosaccharides is represented by the sugar residues in open type. The possible outer arm residues are bracketed. All possible combinations are observed. SA = Sialic acid; G = galactose; GN = N-acetylglucosamine; M = mannose; F = fucose. N-linked attachment of oligosaccharide occurs on the amide side chain of the Asn-x-Ser/Thr sequon ( $x \neq \text{Pro}$ ); the Ser/Thr residue forms hydrogen bond(s) with the amide group in order to activate it for attachment to the primary N-acetylglucosamine residue of the dolichol intermediate, by oligosaccharyltransferase.

was directly related to the ability of an IgG molecule to activate complement; rather than indirectly by allowing access to interaction sites in the C<sub>H</sub>2 and/or C<sub>H</sub>3 domains for effector ligands [4]. The validity of these conclusions has recently been re-evaluated by the application of protein engineering techniques in an attempt to introduce rational structural changes predicted to affect biological activity. The results of these studies demonstrate the necessity for Fc glycosylation and that protein/oligosaccharide interactions determine the generation of a structure that is permissive of Fc-ligand recognition and activation, while failing to confirm a primary role for the hinge region. Our studies suggest that whilst the oligosaccharide moiety may not contribute directly to ligand binding, except for mannan-binding protein, it does exert a subtle influence on protein tertiary and quaternary structure that is essential to 'wild type' activity. Consequently, Fc-ligand recognition, and hence biological activity, may be modulated by judicious replacement of amino acid residues that contribute to non-covalent protein/oligosaccharide interactions.

### Antibody Glycosylation

Human antibody molecules of the IgG class have N-linked oligosaccharide attached at the amide side chain of Asn297 on the  $\beta$ -4 bend of the inner (Fx) face of the C<sub>H</sub>2 domain of the Fc region [5]. The oligosaccharide moiety is of the complex biantennary type having a hexasaccharide 'core' structure (GlcNAc2Man3GlcNAc) and variable outer arm 'non-core' sugar residues, such as fucose, bisecting N-acetylglucosamine, galactose and sialic acid (fig. 1).

Thus, a total of 36 structurally unique oligosaccharide chains may be attached at each Asn297 residue. It is anticipated that glycosylation can be asymmetric so that an individual IgG molecule may have different oligosaccharide chains attached at each of the Asn297 residues within the Fc region such that whilst the heavy chain synthesised within a single antibody-secreting cell may be homogeneous in its amino acid sequence glycosylation can result in the production of  $(36 \times 36)/2 = 648$  structurally unique IgG molecules or glycoforms; NB: the total number of combinations is divided by two because of the two-fold symmetry of the molecule. Analysis of monoclonal and polyclonal IgG demonstrates the presence of all the predicted oligosaccharide species, however, disialylated oligosaccharides may be absent or present at a very low level [6]. With the additional possibility of the presence of complex N-linked oligosaccharide in the Fab region, it is apparent that glycosylation is a post-translational modification that can introduce a very significant structural and, possibly, functional heterogeneity into the IgG molecule. The presence of additional glycosylation sites within the heavy chains of the other Ig isotypes means that the possible number of glycoforms may be orders of magnitude higher [7, 8].

The Fc glycosylation site is a conserved feature for all mammalian IgGs investigated and glycosylation occurs at a homologous position in human IgM, IgD and IgE, molecules, but not in IgA. Human IgM, IgA, IgE and IgD molecules bear additional N-linked oligosaccharide moieties attached to the constant domains of the heavy chains and IgA subclass 1 (IgA1) and IgD proteins also bear multiple O-linked sugars in their extended hinge regions, attached to hydroxyl groups of serine and threonine residues. It has been estimated that ~30% of polyclonal IgG molecules also bear an oligosaccharide moiety within the Fab region. Since the sequences of the constant region of  $\kappa$  and  $\lambda$  light chains and the  $C_{H}1$  domain of the heavy chain do not include a glycosylation sequon, the oligosaccharide of glycosylated Fab regions is due to attachment within either the  $V_L$  or  $V_H$  sequences. Analysis of the DNA sequences of 83 human germline  $V_H$  gene segments revealed five that encoded potential glycosylation sites, however, none of these sequons were observed in 37  $V_H$  protein sequences – detailed analysis to determine whether the germline gene from which these proteins were derived did encode a glycosylation sequon was not attempted. Fifteen of the 37 protein sequences did have potential glycosylation sequons which, it would appear, have resulted from somatic mutation and antigen selection [7, 8]. It has been demonstrated that the structure and function of Fab oligosaccharide can depend on the site of attachment. Thus, monoclonal murine anti-dextran antibodies with a single oligosaccharide attachment site at residues 54, 58 or 60 in complementarity-determining region 2 (CDR2) were shown to have differing antigen-binding activities [9]. A monoclonal human polyreactive autoantibody, secreted by a heterohybridoma cell

line was shown to be glycosylated on both the  $V_L$  and  $V_H$  regions; the  $V_H$  glycosylation site was at residue 75 in framework 3, adjacent to CDR3 [10].

### Structural Consequences of IgG Glycosylation

Whilst glycosylation of the IgG/Fc is essential for optimal expression of effector activities mediated through Fc $\gamma$ R and the C1 component of complement direct interaction of the oligosaccharide moiety with these effector ligands has not been demonstrated. Recently, it was reported, however, that in agalactosylated IgG the oligosaccharide moiety 'flips' out of the inter-C $H_2$  space and the terminal N-acetylglucosamine residues become available to bind and activate mannan-binding protein [11], and consequently the classical complement cascade. By contrast, these residues are not available to the lectin *Bandeireae simplicifolia* II in the native form of agalactosylated IgG but become so on denaturation. Resolution of structure for the oligosaccharide chains in x-ray crystallography demonstrates that it is not freely mobile and has definite conformation. From its attachment point at Asn297, it 'runs forward' towards the C $H_2$ /C $H_3$  interface region and it is estimated that 82 non-covalent interactions between core sugar residues and the outer arm residues of the  $\alpha[1 \rightarrow 6]$  arm may be possible [12]. Together with the sugars of the  $\alpha[1 \rightarrow 3]$  arm, the oligosaccharide fills the available volume between the C $H_2$  domains. It may be anticipated, therefore, that whilst interactions with the Fx face of the protein impose structure on the oligosaccharide chain there is a reciprocal influence of the oligosaccharide on the protein structure.

The structural and functional consequences of Fc glycosylation can be assessed by comparison of glycosylated and aglycosylated forms of IgG. The latter can be generated by production in *Escherichia coli*, growing IgG producing cells in the presence of the glycosylation inhibitor tunicamycin or by protein engineering of the glycosylation sequon. It should be appreciated that IgG produced in *E. coli* or in the presence of tunicamycin will have an asparagine residue at 297 whilst site-directed mutagenesis can introduce any chosen amino acid residue; in the present case, alanine. A more subtle approach is to isolate homogeneous glycoforms for structural and functional studies or to replace individual or combinations of amino acid residues that make contacts with sugar residues. The latter approach may allow a detailed understanding of the oligosaccharide/protein interactions in this molecule, the 'rules' of template direction and its effect on the type of oligosaccharide attached and the generation of mutant molecules with new profiles of biologic function.

A small, localised protein structural change has been detected for aglycosylated human chimeric IgG3 and its Fc fragment by  $^1\text{H-NMR}$ . Previous studies

had allowed assignments for each of the five histidine residues, and their distribution through the Fc makes them suitable probes for detection of localised structural change. Such a change was reported for His268 which is in the vicinity of both the carbohydrate attachment site and the lower hinge binding site on IgG for Fc receptors [13]. A similar spectral difference was observed between a glycosylated IgG1 Fc fragment and the aglycosylated form produced as a recombinant protein in *E. coli* [14]. A structural difference between the lower hinge regions (residues 234–237) of glycosylated and aglycosylated IgG was inferred from the different papain cleavage profiles obtained for glycosylated and aglycosylated mouse IgG2b. Whilst a single cleavage point at residue 229 was observed for the glycosylated protein, the aglycosylated mutant was cleaved heterogeneously at residues 228, 234, and 235 [15].

Recent <sup>13</sup>C-NMR studies have provided direct evidence of differing structural dynamics for the lower hinge residues of glycosylated and aglycosylated mouse IgG2b [Kato, Lund and Jefferis, unpubl.]. Significant differences are revealed when thermodynamic parameters are determined from data obtained from differential scanning microcalorimetry of glycosylated and aglycosylated mouse IgG2b-Fc [Tischenko, Lund and Jefferis, unpubl.]. Differences are observed in both the C<sub>H</sub>2 and C<sub>H</sub>3 domains and the free energy of stabilisation of the C<sub>H</sub>2 domain is decreased. An attempt to monitor structural differences between glycosylated and aglycosylated human IgG3 through altered epitope expression, employing a panel of >30 mouse monoclonal anti-human Fcγ antibodies, did not detect any loss of expression or obvious reduction in affinity.

### **Functional Consequences of Asn297 Glycosylation**

Since it has been consistently demonstrated that glycosylation is essential for optimal expression of Fc<γR- and C1-mediated effector functions, it may be anticipated that biological activity may vary between differing glycoforms. Most studies have compared differences between natural forms of IgG and their aglycosylated or agalactosylated counterparts; however, we have added the approach of generating mutant proteins in which residues reasoned to participate in oligosaccharide-protein interactions have been replaced.

A wide range of effector cells are activated by IgG/antigen immune complexes through interactions with cellular receptors for the Fc region of the gamma chain, Fc<γR. Three types of human Fc receptors (Fc<γRI, Fc<γRII, and Fc<γRIII) have been defined, by gene cloning and sequencing, that are differentially expressed on a variety of cell types; additionally Fc<γR may be induced or their expression up-regulated following cellular activation. The IgG isotype specificity of the Fc<γR suggests that recognition is correlated with

primary amino acid sequence. An earlier prediction that the lower hinge residues 234–237 (-Leu-Leu-Gly-Gly-) in particular might correlate with Fc $\gamma$ RI recognition appeared to be confirmed by protein engineering studies with the demonstration that replacement of any one of these residues in mouse/human chimeric IgG3 affected recognition by all three human Fc $\gamma$ R [16, 17]. It was proposed, therefore, that the three Fc receptors are recognised by overlapping, non-identical ligand-binding sites. This appears rational for a family of receptors that are evolutionarily related and exhibit a high degree of sequence homology.

Other structural features are also determinants for recognition since replacement of Pro331 by serine, the amino acid residue present at this position in IgG4, reduces the binding affinity for IgG1 and IgG3 by an order of magnitude [18]; these residues are within 11 Å of the lower hinge. The Fc $\gamma$ RII receptor is polymorphic and the allelic forms are designated as Fc $\gamma$ RIIa-H131 and Fc $\gamma$ RIIa-R131 to indicate that a histidine/arginine interchange at residue 131 is critical to recognition of human IgG2 molecules [19]. Thus, monocytes of homozygous H/H131 individuals were found to internalise IgG2-opsonized erythrocytes more efficiently than cells from R/R131 individuals. Since the lower hinge region of IgG2 molecules is radically different from that of IgG1 and IgG3, it is apparent that the recognition site for Fc $\gamma$ RII depends on structure outside this region.

The amino acid replacement studies suggest that Fc $\gamma$ R recognition is dependent on a precise molecular architecture and that subtle structural changes have a dramatic effect on biological function. This conclusion is further supported by the demonstration that aglycosylated human chimeric IgG3 has a reduced interaction with all three Fc receptors [16, 17, 20]. Whilst haptens-derivatised red blood cells could still be sensitised with this antibody to trigger superoxide production by U937 cells, stimulated with  $\gamma$ -interferon, higher levels of sensitisation were required compared to glycosylated IgG3 [20]. The aglycosylated IgG3 was not recognised by human Fc $\gamma$ RII expressed on K562 and Daudi cells [21] and rosette formation mediated through Fc $\gamma$ RIII, expressed on human NK (natural killer) cells, was reduced to 40% of that obtained for glycosylated IgG3, whereas antibody-dependent cellular cytotoxicity (ADCC) was essentially abolished [17]. Comparative studies of a glycosylated and an aglycosylated humanised anti-CD3 antibody suggest that the altered biological activities of aglycosylated IgG may be exploited for some in vivo applications. In the model investigated the glycosylated IgG was able to effect immune modulation and was immunogenic; probably due to its ability to activate T cells following interactions with appropriate Fc $\gamma$ R-expressing cells. By contrast, the aglycosylated antibody was not immunomodulatory, was less immunogenic and had a longer half-life [22].

Attempts to evaluate the contribution of outer arm sugars to biologic function have concentrated mostly on glycoforms differing in galactose content. In a sustained investigation of the EBV-transformed lymphocytes secreting anti-D antibody, it has been shown that antibody with a high galactose content (> 70% digalactosyl IgG) was more effective than antibody with a low galactose content (10% agalactosyl and 50% monogalactosyl IgG) in Fc $\gamma$ RI- and Fc $\gamma$ RIII-mediated cellular lysis (ADCC) [23]. An evaluation of the contribution of galactosylation to Fc $\gamma$ RI recognition was made by comparison of the ability of a low galactose (<20% galactosylated) and a fully galactosylated form of an IgG4 Fc to inhibit superoxide generation through mouse/human chimeric IgG3, no difference was detectable in this system [24]. A minimal reduction in Fc $\gamma$ R and a 2-fold reduction in C1q binding for agalactosyl IgG relative to the galactosylated form has been reported [25].

Considerable clinical experience has been gained with the humanised monoclonal antibody Campath-1H and its promise requires optimisation of control and efficiency of production. The product of rat YO, chinese hamster ovary (CHO) and mouse NSO cells has been evaluated for glycosylation and ADCC activity [26]. Interestingly, the rat cells were demonstrated to secrete IgG with relatively high levels of bisecting GlcNAc and to be the most active of the three products in ADCC, leading to the conclusion that this glycoform may have significant biologic advantage. The product of the NSO cells was reported to be underglycosylated. The final conclusion was that the cell type was a more important parameter than the culture conditions, at least for medium with and without added serum. It should be noted, however, that the method of culture used for each cell type was significantly different; the YO cells were grown in roller bottles, the NSO cells in shaking flasks and the CHO cells in hollow fibre bioreactors. In our experience [27], these differences in the method of culture could account, in large part, for the differences in glycoform profiles observed. The influence of outer arm sugars was evaluated for Campath-1H antibody following exposure to neuraminidase and  $\beta$ -galactosidase; removal of low levels of sialic acid had no effect on ADCC or complement-mediated lysis (CML), however, whilst removal of galactose was without effect on ADCC, it resulted in ~50% reduction in CML activity [28].

The essential requirement for protein/core-oligosaccharide interactions with a biantennary-type oligosaccharide is suggested from studies of a chimeric mouse-human IgG1 antibody produced in Lec-1 cells which are incapable of processing high mannose forms of oligosaccharide [29]. The antibody product having a high mannose oligosaccharide attached at Asn297 was incapable of complement-mediated hemolysis and deficient in C1q and Fc $\gamma$ RI binding. In contrast a chimeric mouse-human IgG1 antibody produced in yeast cells, with presumed incorporation of high mannose forms of oligosaccharides at Asn297,

maintained the ability to trigger ADCC through human Fc $\gamma$ RIII [30]. The importance of C<sub>H</sub>2 domain protein/core-oligosaccharide interactions in IgG is emphasised by the demonstration that recognition by Fc $\gamma$  receptors can be modulated in mutant proteins in which core oligosaccharide contact residues have been replaced. Thus, replacement of Asp265, a contact residue for the primary GlcNAc residue of the core oligosaccharide, resulted in reduced recognition by human Fc $\gamma$ RI and human Fc $\gamma$ RII. By contrast, replacement of non-core contact residues Lys246, Asp249 by Ala and Glu258 by Asn was without effect on recognition for these receptors, a finding consistent with the view that the interactions with GlcNAc and Gal residues of the Man $\alpha(1\rightarrow 6)$  arm are not essential for maintenance of recognition by human Fc $\gamma$ RI and Fc $\gamma$ RII [24].

The biological half-life of a recombinant glycoprotein is a vital property determining in vivo efficacy and the economics of treatment. Studies of blood clearance of glycosylated and aglycosylated mouse/human chimeric IgG1 in mice demonstrated accelerated clearance for the aglycosylated form but with similar half-lives. Since the half-life of IgG1 in humans is ~23 days but measured as 5 days in this model it is difficult to draw a definitive conclusion. Catabolism of aglycosylated mouse IgG2b was evaluated in a rat model and shown to be cleared more rapidly than the glycosylated form and it was concluded that the increased catabolism occurred in the extravascular space [31]. The plasma half-lives and bioavailability of human anti-D antibodies secreted by Epstein-Barr virus (EBV)-transformed human B cells, cultured in hollow-fibre bio-reactors, have been evaluated in vivo in comparison with polyclonal anti-D isolated from immunised volunteers [32]. The half-lives of an IgG1 and an IgG3 monoclonal anti-D antibody were 22.2 and 10.2 days, respectively, compared to 15.6 days for polyclonal anti-D IgG. The half-life of polyclonal anti-D IgG was dependent on the proportions of IgG1 and IgG3 present in the preparation. Studies of mutant mouse IgG1 proteins have been interpreted to localise the site controlling catabolism to the inter C<sub>H</sub>2/C<sub>H</sub>3 region and to demonstrate modulation of the half-life [33].

Evaluation of a panel of 28 mutant mouse IgG2b proteins, each with a surface accessible amino acid replacement, for Clq binding and Cl activation correlated recognition with the presence of the wild-type residues lysine, glutamic acid and glutamic acid at 318, 320 and 322 [34]. A contrary result has been reported for mouse/human chimeric IgG1 antibody with the demonstration that replacement of glutamic acid 320 was without effect on CML, however, Cl activation was abrogated following amino acid replacements in the lower hinge region [35]. This is consistent with the observation that a Pro → Ser replacement at residue 331 in IgG1 and IgG3 results in a reduced capacity to trigger complement lysis [36]. One of the mutant proteins produced by Duncan

and Winter [34] was Asn → Ala, 297 which results in the production of aglycosylated mouse IgG2b. This protein had a 3-fold reduced capacity to bind human C1q and a much reduced ability to trigger lysis of target cells with guinea pig complement through the classical complement cascade. Similarly, an aglycosylated mouse/human chimeric IgG1 was shown to retain some ability to trigger lysis of target cells by human complement but with a 7- to 8-fold higher antibody concentration requirement than for the glycosylated wild type IgG1 [37]. These data suggest a similarity in the molecular requirements for Fc<sub>y</sub>R and C1 recognition and that glycosylation is essential for generation of a quaternary structure expressing these ligand binding sites.

The role of outer arm sugars in C1 mediated lysis has been investigated for galactosylated and agalactosylated IgG, produced following exposure to β-galactosidase, with an observed 2-fold higher activity for the galactosylated form [25]. Confirmation of the importance of correct glycosylation is provided by study of a human-mouse chimeric IgG1 molecule produced in yeast cells and anticipated to have high mannose type oligosaccharide attached at Asn297 [29]. The IgG1 product was unable to activate C1 to trigger human complement mediated lysis of targets whilst the same chimeric IgG1 construct expressed in rodent cells (Sp2/0) was effective. A direct role for the oligosaccharide moiety in activating the complement cascade is apparent for the lectin mannan-binding protein which can function as a surrogate C1 component. The specificity of mannan-binding protein is for mannose and N-acetylglucosamine residues, and it has been shown that it can access and bind to terminal N-acetylglucosamine residues exposed on agalactosyl IgG [11].

Much interest has been generated by the observation of a deficit in IgG galactosylation in patients with rheumatoid arthritis (RA) and some other inflammatory diseases, including tuberculosis and Crohn's disease [38]. Another feature of RA is the presence in the blood of rheumatoid factor (RF) autoantibodies having specificity for epitopes in the Fc region of IgG. Since RFs are, typically, of IgM or IgG isotype, the immune complexes formed have the potential to trigger effector functions through IgG-mediated pathways or a combination of IgG and IgM pathways. The chronic inflammatory reactions resulting are thought to contribute erosive damage in this disease. A dominant specificity of RFs is for an epitope localised to the area of contact and interaction between the C<sub>H</sub>2 and C<sub>H</sub>3 domains. This specificity overlaps with that of Staphylococcal protein A and the binding of a majority of RFs to IgG can be inhibited by Staphylococcal protein A [8, 39]. It has been speculated that terminal galactose or sialic acid residues on the α[1 → 6] arm of the oligosaccharide may be accessible to RFs and influence recognition and binding affinity, with a consequent effect on the nature and size of immune complex formed. A galactose residue on the α[1 → 6] arm is resolved on x-ray crystallography

and possible non-covalent contacts identified. It has been argued, therefore, that this galactose residue occupies a lectin-like pocket that will be exposed in agalactosylated IgG and may contribute to altered IgG antigenicity, e.g. reactivity with RFs [38]. Alternatively, the mannose-binding protein provides a route by which agalactosylated IgG could trigger the inflammatory reactions seen in RA independently of RFs [11].

A study of the reactivity of 16 monoclonal RFs generated from synovial tissue lymphocytes with IgG of differing galactose content (18–86%) yielded ambiguous results. Five RFs reacted more avidly to IgG of low galactose content, 6 were not influenced by galactose content and one bound more avidly to IgG of high galactose content [40]. A comparison of the binding of polyclonal and monoclonal RFs to glycosylated and aglycosylated chimeric mouse/human IgG proteins of each of the subclasses detected no differences for IgG1, IgG2 and IgG4 proteins, however, RFs reactive with IgG3 proteins reacted more avidly with aglycosylated IgG3 [41]. In a companion study, some monoclonal RFs were found to bind aglycosylated IgG4 less well than glycosylated IgG4 (2- to 5-fold), suggesting that the carbohydrate moiety is important in establishing their binding epitope in the C<sub>H</sub>2 domain [42]. An interesting difference between the latter two studies was that for one the source of monoclonal RF was serum of patients with Waldenströms macroglobulinemia [41] and for the other EBV-transformed synovial tissue lymphocytes of RA patients. Given the parallelism between RF and Staphylococcal protein A binding to IgG, it is pertinent to note that there is only one report of a minimal effect of glycosylation on the binding of Staphylococcal A to IgG. An interesting recent study demonstrated isotype regulation mediated through the generation of auto-anti-isotype antibodies (RFs) during the course of an immune response to influenza virus. A series of RFs were established as monoclonal antibodies and demonstrated to effect immune deviation *in vivo*. One of these RFs, a monoclonal IgA RF, specific for mouse IgG2b bound the aglycosylated protein poorly [43].

On complexing with polyvalent antigen, IgM is able to initiate the classical complement cascade, following binding of C1q molecule to the C<sub>H</sub>3 domain; the equivalent of the C<sub>H</sub>2 domain of IgG. Amino acid replacements within glycosylation sequon 402–404 [44] of the C<sub>H</sub>3 domain of mouse IgM results in a 3- to 25-fold decrease in the capacity to effect CML of target cells by guinea pig complement. This lowered activity could be due, at least in part, to an observed 4- to 8-fold reduction in assembly of the monomeric subunits into pentameric and hexameric IgM molecules. Replacement of residue 406 (Ser→Asn), analogous to core contact residue 301 in IgG, resulted in a 50-fold decrease in the capacity of mouse IgM to trigger lysis through guinea pig complement [45]. These data suggest that interactions between amino acid

residues and core sugar residues of the oligosaccharide attached at Asn-402 of the IgM molecule may be important for the formation of the Cl-binding and activation site.

### **Factors Influencing Glycosylation Hybridoma, and Recombinant Immunoglobulin Molecules**

Regulatory authorities demand exhaustive testing of monoclonal antibodies that might be applied for in vivo diagnostic or therapeutic purposes. If approved, a similar demand for the demonstration of product consistency is made. The parameters that should be analysed, *in vitro*, include isotype, subclass, affinity, microheterogeneity, molecular weight, primary and secondary structure, structural integrity, specificity, glycosylation profile, biological potency. Subsequently, the product would be evaluated for pharmacological, toxicological, biodistribution and half-life *in vivo* [3]. Functional studies of recombinant human proteins have established that the form of the oligosaccharide moiety attached at a specific glycosylation site should be the same as that attached to the natural molecule. Regulatory authorities require authentic and consistent glycosylation of molecules that may be applied *in vivo*, therefore, animal cells are preferred to other systems for their production. The biotechnology industry has concentrated on development of production protocols employing CHO cells for all recombinant human glycoproteins demonstrating that its glycosylation machinery is catholic and that the polypeptide chain has a major influence on the type of oligosaccharide attached. However, CHO cells do not satisfy industrial economic requirements for the production of antibodies and so there has been a resurgence of interest in NSO cells that are derived from an antibody secreting plasmacytoma. A recent study also employed the rat plasmacytoma line YO and demonstrated its product to have a natural glycosylation profile that included the presence of bisecting GlcNAc residues [26]. For any given cell type, glycosylation of antibody products remains a variable dependent on numerous parameters that include, the method of cell culture, the supply of nutrients, removal of metabolic products, when the protein is harvested and a subtle influence of the polypeptide chain on outer arm sugar heterogeneity. A further concern is the possibility that mutant clones may arise during extensive and continuous culture with the emergence and overgrowth of a sub-clone secreting structurally and functionally aberrant molecules. The reality of this concern is demonstrated by the isolation of multiple sub-clones of CHO cells each of which expresses an altered profile of glycosyltransferases and consequently secretes glycoproteins with unique glycoform profiles [46].

Experience in the production of mouse/human chimeric antibodies in J558L cells demonstrated significant differences in galactosylation depending on whether it was produced in shallow culture, hollow fibre bioreactor or in vivo, as ascitic fluid. Of particular concern is the production of variable proportions of molecules bearing additional galactose residues in  $\alpha[1 \rightarrow 3]$  linkage to normal galactose sugars. This results from the activity of an endogenous  $\alpha[1 \rightarrow 3]$ -galactosyltransferase. Gene expression for this enzyme is depressed in humans and higher primates with the result that it constitutes an immunogenic structure and it has been estimated that 1% of circulating human IgG is 'anti-Gal' antibody [47] and its presence can be readily demonstrated in an ELISA [48]. Although the CHO cell line expresses an  $\alpha[1 \rightarrow 3]$  galactosyltransferase, there appears to be only one documented instance in which it has been demonstrated to be active in the addition of Gal  $\alpha[1 \rightarrow 3]$  Gal [49]. We have had the experience of culturing multiple clones of transfected J558L whose antibody product had an essentially normal glycoform profile over several years; then, for reasons unknown to us, the antibody product included high mannose oligosaccharides and low site occupancy [Lund, Takahashi and Jefferis, unpubl. obs.]. A similar experience has been reported for human IgG1 and IgG2 antibodies produced by heterohybridomas for which variable proportions of high mannose containing antibody was obtained [50]. Glycosylation appeared to be dictated by the mouse plasmacytoma partner since N-glycolylneuraminic acid but no bisecting GlcNAc was added. Similarly, a humanised anti-CD18 antibody produced in NSO cells was shown to contain five oligomannoside-type structures in addition to the usual biantennary-type oligosaccharide moieties, no bisecting GlcNAc and no sialic acid [51]. The glycosylation status of human anti-D antibody produced by EBV-transformed lymphocytes grown at low density in static culture or high density in hollow fibre bioreactors also demonstrated high levels of galactosylation for antibody produced at low density and a relatively natural profile of glycoforms for antibody produced in the bioreactor [23]. Heterohybridomas secreting anti-D antibody have also been established; however, analysis showed that 12/16 such cell lines had incorporated Gal $\alpha[1 \rightarrow 3]$ Gal epitopes into the antibody [52].

A further rodent/human difference is in the form of sialic acid utilised. Polyclonal human IgG has a terminal N-acetyl neuraminic acid sugar on ~25% of oligosaccharides, by contrast the mouse utilises N-glycolyl neuraminic acid. Interestingly, chimeric mouse/human IgG3 produced in J558L cells was shown to be a mixture of molecules having one or the other derivative. This demonstrates that both transferases are available and that their utilisation is affected by subtle structural effects. A bisecting GlcNAc residue is present in ~10–20% human polyclonal IgG but NSO and CHO cells lack the GlcNAc transferase III enzyme required for its addition. The extensive functional

studies reported for recombinant molecules produced in CHO cells suggested that bisecting GlcNAc has little influence on biological activity, however, the presence of glycoforms with bisecting GlcNAc produced by rat YO cells has been held to account for its beneficial biological activity [26]. These findings point to the need for a productive cell line of human origin, however, none is available that has a high endogenous rate of protein synthesis. Many other vehicles for recombinant protein production are being appraised or under development. The early promise of *E. coli* has not been realised for glycoproteins since bacteria do not have a glycosylation machinery [14]. Experiences with insect cells differ. Thus whilst a mixture of high mannose and complex N-linked oligosaccharides was reported for recombinant human plasminogen, including a fully elongated biantennary form (28%) [53] the conclusion drawn from a study of the N-glycosylation of a virion protein was that insect cells were not capable of elongation with the addition of galactose and sialic acid [54]. It is evident that this system is very sensitive to culture conditions and the timing of infection with baculovirus. It is unlikely that other expression systems, such as transfected potatoes, tomatoes, are likely to allow production of glycoproteins that will be acceptable for therapeutic use; it should be remembered that glycosylation is only one of several post-translational events that are essential to the synthesis of a natural form of proteins and glycoproteins.

Whilst one might attempt to develop optimal growth conditions for basic scientific studies, it is likely that they would be too costly to translate into commercial production protocols. Ideally, one would aim for a system that mimics *in vivo* conditions (homeostasis!) as closely as possible with the maintenance of nutrient concentrations, oxygen tension, removal of metabolites. An unknown factor is the presence of essential growth factors (cytokines) *in vivo*. A major consideration for biotechnology companies is the overall cost of production and an important element in its determination is downstream processing. Isolation and purification is simplified by the use of defined media and there has been a sustained development of serum-free media with most companies adopting their own undisclosed formulation. Large-scale production facilities have employed air-lift fermenters of 10–12,000 litres capacity. There is a gradual scale-up with the growth of a 'charge' for the next fermenter to allow exponential growth. At the end stage, the cells exhaust the medium, die and protein is released following rupture of the cell wall.

Hollow-fibre bio-reactors have been used for research and intermediate scale production of glycoproteins, including antibodies to be used as *in vivo* therapeutics. This system does allow continuous exchange between the medium that the cells are suspended in and the 'external' circulating medium. However, the cells are not homogeneously dispersed throughout the cell compartment

but grow in clumps of solid tissue with the result that mass transfer across such a tissue is inefficient and necrosis follows.

We have commented on the heterogeneity of glycosylation of IgG produced in vivo by healthy human adults and instances altered galactosylation patterns in certain inflammatory diseases. It remains to be determined whether the IgG producing plasma cells are or are not abnormal per se or are developing and producing IgG in an abnormal environment. A fundamental question is whether this is a disease-specific phenomenon that has direct implications for cause and progression or an epiphomenon that may be used to monitor disease activity and may have value as a prognostic indicator, as for  $\alpha$ -acid glycoprotein [55]. Analysis of mouse and human monoclonal IgGs has demonstrated that each clone exhibits a unique Fc glycosylation profile and, therefore, that the profile for polyclonal IgG is the sum of the many contributing clones. The human monoclonal IgG proteins have been isolated from sera of patients with the disease multiple myeloma. Analysis of a panel of IgG paraproteins with multiple examples of each subclass revealed a subtle template direction effect such that the apparent preference, in polyclonal IgG, for galactosylation of the  $\alpha[1 \rightarrow 6]$  arm over the  $\alpha[1 \rightarrow 3]$  arm was reversed for IgG2 proteins and for 2/3 IgG3 proteins [6]. In an extension of these studies, we have observed the  $\alpha[1 \rightarrow 3]$  preference for a further five IgG3 paraproteins whilst the polyclonal IgG isolated from the same serum sample demonstrated the opposite preference [56]. A further observation is hypogalactosylation of both the polyclonal and the monoclonal IgG, relative to normal polyclonal IgG; however, this did not correlate with IL-6 levels in the same serum sample. It has been shown that IL-6 is a growth factor for plasma cells proliferating in the bone marrow in this disease and that it is reflected in increased IL-6 levels in the serum [57].

For proteins with multiple glycosylation sites, fidelity is observed for the type of oligosaccharide attached at each. Such template direction, excluding outer arm sugars, is exemplified for IgM, IgA, IgE and IgD molecules. Thus, for a mouse IgM secreting plasmacytoma, the oligosaccharide attached at Asn171 is a biantennary complex form, oligosaccharides at Asn332, 364 and 402 are triantennary and at Asn563 predominantly chitobiose ( $\text{Man}_3\text{GlcNac}_2$ ) [58]. Similarly, in the human IgD molecule an oligomannose form is attached at residue 354 within the C<sub>H</sub>2 domain, and complex forms at residues 445 and 496 within the C<sub>H</sub>3 domain [59, 60]. While large-scale steric effects have been invoked in influencing accessibility of glycosylation sites to glycosylation enzymes, it is less widely appreciated that smaller-scale changes can also affect glycan synthesis. Repositioning of a carbohydrate attachment site within the Fab region of an antidextran antibody by two residues from Asn58 to Asn60 [9], resulted in the attachment of oligomannose forms in place of a complex form and was accompanied by  $\geq 3$ -fold reduction in affinity for antigen. This

study employed mouse hybridoma cells for antibody production and noted that the Fab oligosaccharide was more fully processed than the Fc moiety and that Gal $\alpha$ [1 → 3]Gal was added to a significant proportion of Fab oligosaccharide. A more subtle influence of glycosylation status has been demonstrated for a human hybridoma that has a glycosylated  $\lambda$ -chain; variations in glucose availability were shown to determine the size of the oligosaccharide attached and the antigen-binding activity [61]. Alternatively, it has been shown that glycosylation sequons can be introduced into variable regions with consequent glycosylation that does not affect antigen binding and which can be used for conjugation of haptens [62].

The extensive interactions between the oligosaccharide and protein moieties suggest the possibility to modulate them by selected amino acid replacements with a consequent influence on biological activity. Such an effect has been observed on replacement of the core contact residue Asp265 by Ala (DA265), resulting in greatly increased levels of galactosylation but a diminution of Fc $\gamma$ RI-mediated function; 50% more oligosaccharide chains with galactose residues on both the  $\alpha$ [1 → 3] and  $\alpha$ [1 → 6] arms for mouse/human chimeric IgG3 produced in CHO cells [63].

In conclusion, it is evident that post-translational glycosylation of proteins can have subtle and more far-reaching structural and functional consequences. These consequences will be particularly manifest for recombinant glycoproteins produced *in vitro* but intended for *in vivo* application. A second rapidly developing area of interest results from the observation of altered glycosylation states for specific proteins correlating with disease and/or disease activity. The field is set to expand as sensitive technologies for determining oligosaccharide structures and profiles are now commercially available.

## References

- 1 Dwek R: Glycobiology – towards understanding the function of sugars. *Biochem Soc Trans* 1995; 23:1–25.
- 2 Jenkins N, Curling E: Glycosylation of recombinant proteins: Problems and prospects. *Enzyme Microb Technol* 1995;16:354–364.
- 3 Schaffner G, Haase M, Geiss S: Criteria for investigation of the product equivalence of monoclonal antibodies for therapeutic and *in vivo* diagnostic use in case of change in manufacturing process. *Biologicals* 1995;23:253–259.
- 4 Burton D, Woof JM: Antibody effector functions. *Adv Immunol* 1992;52:1–48.
- 5 Beale D, Feinstein A: Structure and function of the constant regions of immunoglobulins. *Q Rev Biophys* 1976;9:135–180.
- 6 Jefferis R, Lund J, Mizitani H, Nakagawa H, Kawazoe Y, Arata Y, Takahashi N: A comparative study of the N-linked oligosaccharide structures of human IgG subclass proteins. *Biochem J* 1990; 268:529–537.

- 7 Jefferis R: Glycosylation of antibody molecules: functional significance. *Glycoconj J* 1993;10: 357-361.
- 8 Jefferis R, Lund J, Goodall M: Recognition sites on human IgG for Fc<sub>y</sub> receptors: The role of glycosylation. *Immunol Lett* 1995;44:111-117.
- 9 Endo T, Wright A, Morrison SL, Kobata A: Glycosylation of the variable region of immunoglobulin G-site-specific maturation of the sugar chains. *Mol Immunol* 1995;32:934-940.
- 10 Leibiger H, Hansen A, Schoenherr G, Seifert M, Wustner D, Stigler R, Marx U: Glycosylation analysis of a polyreactive human monoclonal IgG antibody derived from a human-mouse heterohybridoma. *Mol Immunol* 1995;32:595-602.
- 11 Malhotra R, Wormald MR, Rudd PM, Fischer PB, Dwek RA, Sim RB: Agalactosyl IgG activates complement via mannose-binding protein. *Nat Med* 1995;1:237-243.
- 12 Padlan E: x-Ray diffraction studies of antibody constant regions, in Metzger H (ed): *Fc Receptors and the Action of Antibodies*. Washington, American Society for Microbiology, 1990, pp 12-30.
- 13 Lund J, Tanaka T, Takahashi N, Sarmay G, Arata Y, Jefferis R: A protein structural change in aglycosylated IgG3 correlates with loss of huFcRI and huFcRIII binding and/or activation. *Mol Immunol* 1990;27:1145-1153.
- 14 Matsuda H, Nakamura S, Ichikawa Y, Kozai K, Takano R, Nose M, Endo S, Nishimura Y, Arata Y: Proton nuclear magnetic resonance studies of the structure of the Fc fragment of human immunoglobulin G1: Comparisons of native and recombinant proteins. *Mol Immunol* 1990;27:571-579.
- 15 Hindley S, Gao Y, Nash P, Sautes C, Lund J, Goodall M, Jefferis R: The interaction of IgG with Fc<sub>y</sub>RII: Involvement of the lower hinge binding site as probed with NMR. *Biochem Soc Trans* 1993;21:337S.
- 16 Lund J, Winter G, Jones PT, Pound JD, Tanaka T, Walker MR, Artymuk PJ, Arata Y, Burton DR, Jefferis R, Woof JM: Human Fc<sub>y</sub>RI and Fc<sub>y</sub>RII interact with distinct but overlapping binding sites on the human IgG. *J Immunol* 1991;147:2657-2662.
- 17 Sarmay G, Lund J, Rozsnyay Z, Gergely J, Jefferis R: Mapping and comparison of the interaction sites on the Fc region of IgG responsible for triggering antibody dependent cellular cytotoxicity (ADCC) through different types of human Fc receptor. *Mol Immunol* 1992;29:633-639.
- 18 Canfield SM, Morrison SL: The binding affinity of human IgG for its high affinity Fc receptor is determined by multiple amino acids in the C<sub>H</sub>2 domain and is modulated by the hinge region. *J Exp Med* 1991;173:1483-1491.
- 19 Warmerdam PAM, van de Winkle JGJ, Gosseli EJ, Capel PJA: A single amino acid in the second domain of the human Fc<sub>y</sub> receptor II is critical for human IgG2 binding. *J Immunol* 1991;172: 19-25.
- 20 Pound JD, Lund J, Jefferis R: Aglycosylated chimeric human IgG3 can trigger the human phagocyte respiratory burst. *Mol Immunol* 1993;30:233-241.
- 21 Walker MR, Lund J, Thompson KM, Jefferis R: Aglycosylation of human IgG1 and IgG3 monoclonal antibodies can eliminate recognition by human cells expressing Fc<sub>y</sub>RI and Fc<sub>y</sub>RII receptors. *Biochem J* 1989;259:347-353.
- 22 Routledge EG, Falconer ME, Pope H, Lloyd IS, Waldmann H: The effect of aglycosylation on the immunogenicity of a humanized therapeutic CD3 monoclonal antibody. *Transplantation* 1995;60: 847-853.
- 23 Kumpel BM, Rademacher TW, Rook GAW, Williams PJ, Wilson IBH: Galactosylation of human IgG monoclonal anti-D produced by EBV-transformed B-lymphoblastoid cell lines is dependent on culture method and affects Fc receptor-mediated functional activity. *Hum Antibody Hybridomas* 1994;5:143-151.
- 24 Lund J, Takahashi N, Pound JD, Goodall M, Nakagawa H, Jefferis R: Oligosaccharide-protein interactions in IgG can modulate recognition by Fc<sub>y</sub> receptors. *FASEB J* 1995;9:115-119.
- 25 Tsuchiya N, Endo T, Matsuta K, Yoshioka S, Aikawa T, Kosuge E, Takeuchi F, Miyamoto T, Kobata A: Effects of galactose depletion from oligosaccharide chains on immunological activities of human IgG. *J Rheumatol* 1989;16:285-290.
- 26 Lifely M, Hale C, Boyce S, Keen MJ, Phillips J: Glycosylation and biological activities of Campath-1H expressed in different cell lines and grown under different culture conditions. *Glycobiology* 1995; 5:813-822.

- 27 Lund J, Takahashi N, Nakagawa H, Goodall M, Bentley T, Hindley SA, Tyler R, Jefferis R: Control of IgG/Fc glycosylation: A comparison of oligosaccharides from chimeric human/mouse and mouse subclass immunoglobulin Gs. *Mol Immunol* 1993;30:741-748.
- 28 Boyd PN, Lines AC, Patel AK: The effect of the removal of sialic acid, galactose and total carbohydrate on the functional activity of Campath-1H. *Mol Immunol* 1995;32:1311-1318.
- 29 Wright A, Morrison S: Effect of altered C<sub>n</sub>2 associated carbohydrate structure on the functional properties and in vivo fate of human immunoglobulin 1. *J Exp Med* 1994;180:1087-1096.
- 30 Horwitz AH, Chang CP, Better M, Hellstrom KE: Secretion of functional antibody and Fab fragment from yeast cells. *Proc Natl Acad Sci USA* 1988;85:8678-8682.
- 31 Wawrzynczak EJ, Cumber AJ, Parnell GD, Jones PT, Winter G: Blood clearance in the rat of a recombinant mouse monoclonal antibody lacking the N-linked oligosaccharide side chains of the C<sub>n</sub>2 domains. *Mol Immunol* 1992;219:213-220.
- 32 Kim JK, Tsen MF, Ghetie V, Ward ES: Evidence that the hinge region plays a role in maintaining serum levels of the murine IgG1 molecule. *Mol Immunol* 1995;32:467-475.
- 33 Goodrick J, Kumpel B, Pamphilon D, Fraser I, Chapman G, Dawes B, Anstee D: Plasma half-lives and bioavailability of human monoclonal Rh D antibodies BRAD-3 and BRAD-5 following intramuscular injection into RhD-negative volunteers. *Clin Exp Immunol* 1994;98:17-20.
- 34 Duncan AR, Winter G: The binding site for C1q on IgG. *Nature* 1988;332:738-740.
- 35 Morgan A, Jones ND, Nesbitt AM, Chaplin L, Bodmer MW, Emtage JS: The N-terminal end of the C<sub>n</sub>2 domain of chimeric human IgG1 anti-HLA-DR is necessary for C1q, Fc<sub>y</sub>RI and Fc<sub>y</sub>RII binding. *Immunology* 1995;86:319-324.
- 36 Tao MH, Smith RIF, Morrison SL: Structural features of human immunoglobulin G that determine isotype-specific differences in complement activation. *J Exp Med* 1993;178:661-667.
- 37 Dorai H, Mueller BM, Reisfeld RA, Gillies SD: Aglycosylated chimeric mouse/human IgG1 antibody retains some effector function. *Hybridoma* 1991;10:211-217.
- 38 Parekh RB, Dwek RA, Sutton JB, Fernandes DL, Leung A, Stanworth D, Mizuochi T, Taniguchi T, Matsuta K, Takeuchi F, Nagano Y, Miyamoto T, Kobata A: Association of rheumatoid arthritis and primary osteoarthritis with changes in the glycosylation pattern of total serum IgG. *Nature* 1985;316:452-457.
- 39 Jefferis R, Nik Jaafar MIB, Steinitz M: Immunogenic and antigenic epitopes of immunoglobulins. VIII. A human monoclonal rheumatoid factor having specificity for a discontinuous epitope determined by histidine/arginine interchange as residue 435 of immunoglobulin G. *Immunol Lett* 1984; 7:191-194.
- 40 Soltys AS, Hay FC, Bond A, Axford AS, Jones MG, Randen I, Thompson KM, Natvig JB: The binding of synovial tissue derived monoclonal human immunoglobulin M rheumatoid factors to immunoglobulin G preparations of differing galactose content. *Scand J Immunol* 1994;40:135-143.
- 41 Artandi SE, Canfield SM, Tao MH, Calame KL, Morrison SL, Bonagura VR: Molecular analysis of IgM rheumatoid factor binding to chimeric IgG. *J Immunol* 1991;146:603-610.
- 42 Bonagura VR, Artandi SE, Davidson A, Randen I, Agostino N, Thompson K, Natvig J, Morrison SL: Mapping studies reveal unique epitopes on IgG recognised by rheumatoid arthritis-derived monoclonal rheumatoid factors. *J Immunol* 1993;151:3840-3852.
- 43 Rajnavolgyi E, Fazekas GY, Lund J, Daeron M, Teillaud J, Jefferis R, Fridman W, Gergely J: Activation of effector functions by immune complexes of mouse IgG2a with isotype specific autoantibodies. *Immunology* 1995;85:645-654.
- 44 Muraoka S, Shulman MJ: Structural requirements for IgM assembly and cytolytic activity. Effects of mutations in the oligosaccharide acceptor site at Asn-402. *J Immunol* 1989;142:695-701.
- 45 Shulman MJ, Pennel N, Collins C, Hozumi N: Activation of complement by immunoglobulin M is impaired by the substitution serine-406→asparagine in the immunoglobulin μ heavy chain. *Proc Natl Acad Sci USA* 1986;83:7678-7682.
- 46 Stanley P, Ioffe E: Glycosyltransferase mutants - key to new insights in glycobiology. *FASEB J* 1995;9:1436-1444.
- 47 Galili U, Shohet SB, Kobrin E, Stults CLM, Macher BA: Man, apes and old world monkeys differ from other mammals in the expression of α-galactosyl epitopes on nucleated cells. *J Biol Chem* 1988;263:17755-17762.

- 48 Borraebeck CAK, Malmor AC, Ohlin M: Does endogenous glycosylation prevent the use of mouse monoclonal antibodies as cancer therapeutics? *Immunol Today* 1993;14:477-479.
- 49 Ashford DA, Alaf CD, Gamble VM: Site-specific glycosylation of rat and human soluble CD4 variants expressed in CHO cells. *J Biol Chem* 1993;268:3260-3267.
- 50 Tandai M, Endo T, Sasaki S, Masuho Y, Kochibe N, Kobata A: Structural study of the sugar moieties of monoclonal antibodies secreted by human-mouse hybridoma. *Arch Biochem Biophys* 1991;291:339-348.
- 51 Yu Ip C, Miller WJ, Silberklang M, Mark GE, Ellis RW, Huang L, Glusha J, Van Halbeek H, Zhu J, Alhadeff JA: Structural characterisation of the N-glycans of a humanized anti-CD18 murine immunoglobulin. *G Arch Biochem Biophys* 1994;308:387-399.
- 52 Montano RM, Romano EL: Human monoclonal anti-Rh antibodies produced by human-mouse heterohybridomas express the gal  $\alpha$ (1-3) Gal epitope. *Hum Antibody Hybridomas* 1995;5:152-156.
- 53 Davidson DJ, Castellino FJ: Structures of the Asn-289 linked oligosaccharide assembled on human plasminogen expressed in *Mamestra brassicae* cell line (IZD.MB503). *Biochemistry* 1991;30:6689-6696.
- 54 Jarvis DL, Finn EE: Biochemical analysis of the N-glycosylation pathway in baculovirus-infected insect cells. *Virology* 1995;212:500-511.
- 55 van Dijk W, Turner GA, Mackiewicz A: Changes in glycosylation of acute-phase proteins in health and disease: Occurrence, regulation and function. *Glycosyl Dis* 1994;1:5-14.
- 56 Farooq M, Takahashi N, Arrol H, Drayson M, Jefferis R: Glycosylation of IgG antibody molecules in multiple myeloma. 1st Electronic Glycoscience Conference – proceedings, in press.
- 57 Nakao H, Nishikawa A, Nishiura T, Kanayama Y, Tarui S, Taniguchi N: Hypogalactosylation of immunoglobulin G sugar chains and elevated serum IL-6 in Castleman's disease. *Clin Chim Acta* 1991;197:221-228.
- 58 Anderson DR, Atkinson PH, Grimes WJ: Major carbohydrate structures at five glycosylation sites on murine IgM determined by high resolution  $^1$ H-NMR spectroscopy. *Arch Biochem Biophys* 1985;13:605-618.
- 59 Mellis SJ, Baenziger JU: Structures of the oligosaccharides present at the 3 asparagine-linked glycosylation sites of human IgD. *J Biol Chem* 1983;258:11546-11555.
- 60 Mellis SJ, Baenziger JU: Structures of the O-glycosidically linked oligosaccharides of human IgD. *J Biol Chem* 1983;258:11557-11563.
- 61 Tachibana H, Taniguchi K, Ushio Y, Teruya K, Osada K, Murakami H: Changes of monosaccharide availability of human hybridomas lead to alteration of biological properties of human monoclonal antibody. *Cytotechnology* 1994;16:151-157.
- 62 Leung SO, Losman LMJ, Govindan SV, Griffiths GL, Goldenberg DM, Hansen HJ: Engineering a unique glycosylation site for site-specific conjugation of haptens to antibody fragments. *J Immunol* 1995;154:5919-5926.
- 63 Lund J, Takahashi N, Pound JD, Goodall M, Jefferis R: Multiple interactions of IgG with its core oligosaccharide can modulate recognition by complement and human Fc $\gamma$ RI, and influence the synthesis of its oligosaccharide chains. *J Immunol* 1996, in press.

Prof. Roy Jefferis, Department of Immunology, The Medical School,  
University of Birmingham, Birmingham B15 2TT (UK)

# .....

# **Antibody Engineering**

Volume Editor

*J. Donald Capra, Dallas, Tex.*

43 figures, 1 in color, and 9 tables, 1997

**KARGER**

Basel · Freiburg · Paris · London · New York ·  
New Delhi · Bangkok · Singapore · Tokyo · Sydney

.....

## Chemical Immunology

Formerly published as 'Progress in Allergy'  
Founded 1939 by Paul Kallös

Library of Congress Cataloging-in-Publication Data  
Antibody engineering / volume editor, J. Donald Capra.  
p. cm. — (Chemical immunology; 65)  
Includes bibliographical references and index.  
I. Immunoglobulins — Biotechnology. 2. Protein engineering.  
I. Capra, J. Donald, 1937-. II. Series.  
TP248.65.I49A572 1997  
615'.37—dc20  
ISBN 3-8055-6356-6 (hbk.)

Bibliographic Indices. This publication is listed in bibliographic services, including Current Contents® and Index Medicus.

Drug Dosage. The authors and the publisher have exerted every effort to ensure that drug selection and dosage set forth in this text are in accord with current recommendations and practice at the time of publication. However, in view of ongoing research, changes in government regulations, and the constant flow of information relating to drug therapy and drug reactions, the reader is urged to check the package insert for each drug for any change in indications and dosage and for added warnings and precautions. This is particularly important when the recommended agent is a new and/or infrequently employed drug.

All rights reserved. No part of this publication may be translated into other languages, reproduced or utilized in any form or by any means electronic or mechanical, including photocopying, recording, microcopying, or by any information storage and retrieval system, without permission in writing from the publisher.

© Copyright 1997 by S. Karger AG, P.O. Box, CH-4009 Basel (Switzerland)  
Printed in Switzerland on acid-free paper by Thür AG Offsetdruck, Pratteln  
ISBN 3-8055-6356-6

# **CURRICULUM VITAE**

**Dr. Pablo Umaña**

**GlycArt Biotechnology AG**  
**Einsteinstrasse**  
**Zurich 8093, Switzerland**  
**Email: pablo.umana@glycart.com**

## **PROFILE**

Scientific researcher experienced in molecular biology, biochemistry, immunology, cell biology and gene therapy, with an emphasis in the field of antibody glycosylation. Obtained PhD from the California Institute of Technology (carrying out a major part of the experimental work at the ETH-Zurich). Carried postdoctoral research in gene therapy vectors at the University of Manchester, U.K. Co-founder of GlycArt Biotechnology AG, Zurich, a company spinning out of research carried out at the ETH-Zurich during doctoral studies. Joined GlycArt Biotechnology AG as a full-time employee in 2001 as Chief Scientific Officer.

## **EDUCATION**

- 1993-1998. **California Institute of Technology/ ETH-Zurich.** PhD from Caltech, experimental work carried out at the ETH-Zurich. Thesis: Genetic engineering of protein glycosylation in Chinese hamster ovary cells.
- 1991-1993. **California Institute of Technology.** MSc. Thesis: Genetic engineering of protein glycosylation in the insect cell/baculovirus expression system.
- 1985-1990. **University of Costa Rica.** Licenciatura in Chemical Engineering.

## **ACADEMIC HONORS**

Top Engineering Student Award. University of Costa Rica, 1988.

## **PUBLICATIONS**

Umaña, P., Gerdes, C.A., Stone, D., Davies, J.R.E., Ward, D., Castro, M.G. and Lowenstein, P.R.. 2001. Efficient FLP recombinase enables scalable production of helper-dependent adenoviral vectors with negligible helper-virus contamination. *Nature Biotechnology* 19: 582-585.

Umaña, P., Jean-Mairet, J., and Bailey, J.E. 1999. Tetracycline-Regulated Overexpression of Glycosyltransferases in CHO Cells. *Biotechnology and Bioengineering* 65: 542-549.

Umaña, P., Jean-Mairet, J., Moudry, R., Amstutz, H., and Bailey, J.E. 1999. Engineered Glycoforms of an Anti-Neuroblastoma IgG1 with Optimized Antibody-Dependent Cellular Cytotoxic Activity. *Nature Biotechnology* 17: 176-180.

Sburlati, A., Umaña, P., Prati, E. and Bailey, J.E. 1998. Synthesis of Bisected Glycoforms of Recombinant IFN-Beta by Overexpression of GnTIII in Chinese Hamster Ovary Cells. *Biotechnology Progress* 14: 189-192.

Umaña, P. and Bailey, J.E. 1997. A Mathematical Model of N-linked Glycoform Biosynthesis. *Biotechnology and Bioengineering* 55: 890-908.

Lowenstein, P.R.. Thomas, C.E., Umaña, P., Gerdes, C.A., Verakis, T., Boyer, O., Tondeur S., Klatzmann D. and Castro, M.G. 2002. High-capacity, helper-dependent, "gutless" adenoviral vectors for gene transfer into brain. *Methods in Enzymology* 346: 292-311.

Bailey, J. E., Umaña, P., Minch, S., Harrington, M., Page, M., and Sburlati, A. 1997. Metabolic Engineering of N-linked Glycoform Synthesis Systems in Chinese Hamster Ovary Cells. In: *Animal Cell Technology*, Kluwer, p. 489-494.

Umaña, P., Kellis, J.T. and Arnold, F.H. 1993. Recombinant Protein Stabilization through Engineered Metal-Chelating Sites. In: Biocatalyst Design for Stability and Specificity, ACS Symposium Series 516: 102-108.

#### **SUPERVISORY/TEACHING EXPERIENCE**

##### **Chief Scientific Officer (GlycArt Biotechnology AG, 2001-present)**

Supervision of a Diploma project (ETH-Zürich, 1995-1996).

Teaching assistant for Enzyme Technology practical course (ETH-Zürich, 1995).

Teaching assistant for Experimental Physical Chemistry (1989), Thermodynamics (1988-1991), and Reaction Kinetics and Reactor Design (1990) at the University of Costa Rica.

#### **PERSONAL DETAILS**

Date of Birth: 23.09.67; Nationality: Costa Rican; Marital Status: married.

Fluent in written and spoken English. Mother tongue: Spanish.

## **RESEARCH PROJECTS**

### **2001 – present. Chief Scientific Officer:**

Engineering therapeutic antibody glycosylation

GlycArt biotechnology AG, Zürich.

### **1999 – 2001. Postdoctoral Research:**

Development of a new production system for helper-dependent, fully-deleted adenoviral vectors

Anti-angiogenic gene therapy for intracranial tumors.

Supervisor: Prof. Pedro R. Lowenstein.

University of Manchester, U.K.

### **1993-1998. PhD Project:**

Genetic engineering of protein glycosylation in CHO cells

Supervisor: Prof. James E. Bailey

California Institute of Technology/ETH-Zurich

### **1992-1993. MSc Project:**

Genetic engineering of protein glycosylation in the insect cell/baculovirus expression system

Supervisor: Prof. James E. Bailey

California Institute of Technology

### **1991 Summer Project:**

Recombinant protein stabilization by engineered metal chelating sites

Supervisor: Prof. Frances H. Arnold

California Institute of Technology

# Modulation of Therapeutic Antibody Effector Functions by Glycosylation Engineering: Influence of Golgi Enzyme Localization Domain and Co-Expression of Heterologous $\beta$ 1,4-N-acetylglucosaminyltransferase III and Golgi $\alpha$ -mannosidase II

Claudia Ferrara,<sup>1,2</sup> Peter Brünker,<sup>1</sup> Tobias Suter,<sup>1</sup> Samuel Moser,<sup>1</sup> Ursula Püntener,<sup>1</sup> Pablo Umana<sup>1</sup>

<sup>1</sup>GLYCART biotechnology AG, Wagistrasse 18, CH-8952 Schlieren, Switzerland;  
telephone: +41 044 755 61 61; fax: +41 044 755 61 60;  
e-mail: pablo.umana@glycart.com

<sup>2</sup>Institute of Biotechnology, ETH Zürich, Zürich, Switzerland

Received 21 April 2005; accepted 10 October 2005

Published online 24 January 2006 in Wiley InterScience ([www.interscience.wiley.com](http://www.interscience.wiley.com)). DOI: 10.1002/bit.20777

**Abstract:** The effector functions elicited by IgG antibodies strongly depend on the carbohydrate moiety linked to the Fc region of the protein. Therefore several approaches have been developed to rationally manipulate these glycans and improve the biological functions of the antibody. Overexpression of recombinant  $\beta$ 1,4-N-acetylglucosaminyltransferase III (GnT-III) in production cell lines leads to antibodies enriched in bisected oligosaccharides. Moreover, GnT-III overexpression leads to increases in non-fucosylated and hybrid oligosaccharides. Such antibody glycovariants have increased antibody-dependent cellular cytotoxicity (ADCC). To explore a further variable besides overexpression of GnT-III, we exchanged the localization domain of GnT-III with that of other Golgi-resident enzymes. Our results indicate that chimeric GnT-III can compete even more efficiently against the endogenous core  $\alpha$ 1,6-fucosyltransferase ( $\alpha$ 1,6-FucT) and Golgi  $\alpha$ -mannosidase II (ManII) leading to higher proportions of bisected non-fucosylated hybrid glycans ("Glyco-1" antibody). The co-expression of GnT-III and ManII led to a similar degree of non-fucosylation as that obtained for Glyco-1, but the majority of the oligosaccharides linked to this antibody ("Glyco-2") are of the complex type. These glycovariants feature strongly increased ADCC activity compared to the unmodified antibody, while Glyco-1 (hybrid-rich) features reduced complement-dependent cytotoxicity (CDC) compared to Glyco-2 or unmodified antibody. We show that apart from GnT-III overexpression, engineering of GnT-III localization is a versatile tool to modulate the biological activities of antibodies relevant for their therapeutic application.

© 2006 Wiley Periodicals, Inc.

Tobias Suter's present address is Section of Clinical Immunology, University Hospital, CH-8044 Zürich, Switzerland.

Correspondence to: P. Umana

**Keywords:** glycosylation engineering; antibody-dependent cellular cytotoxicity (ADCC); complement-dependent cytotoxicity (CDC); therapeutic IgG; chimeric GnT-III

## INTRODUCTION

Antibodies of the IgG class have proven to be useful anti-cancer therapeutics (Carter, 2001). Their high specificity for an antigen, for targeting a cancerous cell, and the simultaneous recruitment of immune effector cells, by binding to Fc $\gamma$  receptors (Fc $\gamma$ Rs) via their Fc region, make them a powerful tool for immunotherapies. This linker function of the antibodies results in the elimination of the cancerous cell by cell-mediated effector functions, such as antibody-dependent cellular cytotoxicity (ADCC).

Many therapeutic antibodies depend on Fc-mediated effector functions and it was concluded that they require a higher *in vivo* efficacy to increase their potential as therapeutic drugs. The recruitment of Fc $\gamma$ R-expressing cells relies on an efficient binding to the Fc region of IgG (Dall'Ozzo et al., 2004). The affinity of this interaction can be improved by amino acid mutations of the polypeptide (Shields et al., 2001), which bear the risk of immunogenicity. On the other hand the presence of specific oligosaccharide structures linked to the C2 domain of the Fc fragment was reported to affect the biological activity of the antibody (Jefferis et al., 1998; Lifely et al., 1995; Wright and Morrison, 1997) by influencing the interaction with Fc $\gamma$ Rs (Tao and Morrison, 1989). In this context, modification of the carbohydrate moiety associated to the Fc region of IgG has proven to be a successful approach to

enhance ADCC (Shields et al., 2002; Shinkawa et al., 2003; Umaña et al., 1999).

Recombinant DNA-based glyco-engineering for increased antibody effector function was first achieved by over-expression of heterologous  $\beta$ 1,4-N-acetylglucosaminyltransferase III (GnT-III), in antibody-producing cells (Umaña et al., 1999). GnT-III catalyzes the addition of a bisecting N-acetylglucosamine (GlcNAc) to N-linked oligosaccharides, as long as they have been modified by N-acetylglucosaminyltransferase I (GnT-I) and have not been modified by  $\beta$ 1, 4 galactosyltransferase (GalT). Therefore, any non-galactosylated hybrid or complex oligosaccharide, whether fucosylated or not, can be modified by GnT-III. However, once GnT-III adds a bisecting GlcNAc to an oligosaccharide, other central reactions of the biosynthetic pathway such as core-fucosylation and conversion of hybrid to complex glycans are blocked (Schachter, 1986). This gives GnT-III a high degree of control over the glycosylation process in the Golgi apparatus. Overexpression of GnT-III in antibody-producing cells results in the formation of bisected, non-fucosylated oligosaccharides linked to the antibodies that mediate increased ADCC (Shields et al., 2002; Shinkawa et al., 2003; Umaña et al., 1999).

Previously, we have shown that the GnT-III expression level has a large impact on the relative levels of complex and hybrid, fucosylated, or non-fucosylated oligosaccharides (Umaña et al., 1999). Besides the expression level, the Golgi localization domain of GnT-III, which controls its spatial distribution relative to other enzymes, is another variable influencing the impact of GnT-III on the glycosylation pathway (Nilsson et al., 1996). Here we explore the localization variable by fusing the catalytic domain of GnT-III to the localization domain (cytoplasmic, transmembrane, and stem region) of other Golgi-resident enzymes of the N-glycosylation pathway. The resulting chimeric proteins were expressed in antibody-producing cells to engineer the antibody glycosylation pattern and the associated antibody effector functions.

## MATERIALS AND METHODS

### Construction of Expression Vectors

The DNA for the variable heavy (VH) and variable light (VL) chain of the anti-CD20 antibody was assembled by polymerase chain reaction (PCR) on the basis of the published sequence of the murine C2B8 antibody (Kobayashi et al., 1997; Reff et al., 1994). The IgG1 constant regions were amplified from a human leukocyte cDNA library (BD Biosciences, Allschwil, Switzerland). The rat *GnT-III* gene was amplified using specific primers from a rat kidney cDNA library (BD Biosciences) and a sequence coding for a C-terminal c-myc-epitope tag was added. The construction of the GnT-III-chimeric genes was performed by subsequent overlapping PCR reactions. The DNA fragments coding for the localization domains (cytoplasmic, transmembrane, and stem regions) of human GnT-I (102 amino acids), ManII

(100 amino acids), GnT-II (103 amino acids), and  $\alpha$ 1, 6-fucosyltransferase ( $\alpha$ 1,6-FucT) (101 amino acids) were amplified from different material of human origin using the specific primers. The gene coding for Golgi  $\alpha$ -mannosidase II was amplified by PCR from human DNA using specific primers. The gene coding for the human N-acetylglucosaminyltransferase II (GnT-II) was amplified from pGnTII (RG002551, Invitrogen AG, Basel, Switzerland) by PCR using specific primers. The construction of the catalytically inactive GnT-III<sup>ManII</sup> (iGnT-III<sup>ManII</sup>) was performed as described (Ihara et al., 2002). The construction of two mutant GnT-III<sup>mutManII</sup> was accomplished by site-directed PCR mutagenesis, where the mutations R60Q, R73N, L79S, and E81S (GnT-III<sup>mutManII(4aa)</sup>) or R73N, L79S, and E81S (GnT-III<sup>mutManII(3aa)</sup>) were introduced into ManII (Nilsson et al., 1996). All expression vectors were combined with an origin of replication from the Epstein Barr virus (oriP) for episomal vector replication and maintenance in cells producing the Epstein Barr virus nuclear antigen (EBNA). Expression of the protein was confirmed by Western blot detection of GnT-III C-terminal c-myc tag.

For the generation of the Fc $\gamma$ RIIIa-expressing CHO cell line, an expression vector for Fc $\gamma$ RIIIa-Val158  $\alpha$ -chain,  $\gamma$ -chain, and the gene conferring puromycin resistance was constructed. The cDNAs coding for the Fc $\gamma$ RIIIA and the  $\gamma$ -chain were amplified from a healthy donor using specific primers. Genotyping for the Fc $\gamma$ RIIIA-Val/Phe158 and Fc $\gamma$ RIIC polymorphisms were performed as described (Koene et al., 1997; Metes et al., 2001).

### Production and Purification of Glyco-Engineered Anti-CD20 Antibodies in HEK293-EBNA Cells

HEK293-EBNA cells, a kind gift from Rene Fischer (Laboratory of Organic Chemistry, ETH Zürich, Switzerland), were grown as adherent monolayer cultures using DMEM culture medium supplemented with 10% FCS (Invitrogen AG) and were transfected essentially as described by Jordan et al. (1996). HEK293-EBNA cells were used as a transient expression system where the episomal replication of the expression vectors allowed high antibody titres and high expression levels of the glycosylation enzymes. Glyco-engineered antibodies were produced by co-transfection of the cells with two plasmids coding for antibody and chimeric GnT-III, at a ratio of 4:1, respectively, while for unmodified antibody the plasmids coding for the carbohydrate-modifying enzymes were omitted. For the combination of the chimeric GnT-III<sup>ManII</sup> and ManII, cells were co-transfected with three expression vectors coding for antibody, GnT-III<sup>ManII</sup> and ManII at a ratio of 3:1:1. The same ratio was used for the combination of GnT-III and ManII, and for that of ManIIGnT-III and GnT-II. At day 5 post-transfection, supernatant was harvested and monoclonal antibody purified using two sequential chromatographic steps as described (Umaña et al., 1999), followed by size exclusion chromatography (HiLoad<sup>TM</sup> 16/60 Superdex<sup>TM</sup> 200 column, Amersham Biosciences, Otelfingen, Switzerland).

## Oligosaccharide Analysis

Oligosaccharides were enzymatically released from the antibodies by *N*-Glycosidase digestion (PNGaseF, EC 3.5.1.52, QA-Bio, San Mateo, CA) at 0.05 mU/μg protein in 2 mM Tris, pH 7 for 3 h at 37°C. A fraction of the PNGaseF-treated sample was subsequently digested with Endoglycosidase H (EndoH, EC 3.2.1.96, Roche, Basel, Switzerland) at 0.8 mU/μg protein and incubated for 3 h at 37°C. The released oligosaccharides were incubated in mild acid (150 mM acetic acid) prior to purification through a cation exchange resin (AG50W-X8 resin, hydrogen form, 100–200 mesh, BioRad, Reinach, Switzerland) packed into a micro-bio-spin chromatography column (BioRad) as described (Papac et al., 1998). The oligosaccharide samples were then analyzed with sDHB as matrix (Papac et al., 1998) using an Autoflex MALDI/TOF (Bruker Daltonics, Fällanden, Switzerland) in positive ion mode. For the assignment of an oligosaccharide structure to each peak, Endoglycosidase H was used due to its specificity. It digests most hybrid and high mannose, but not complex oligosaccharides. A refined oligosaccharide analysis was necessary to distinguish between bisected hybrid and complex (*m/z* 1,339 and 1,502) and their fucosylated versions (*m/z* 1,648 and 1,810), as both structures are not digested by EndoH. For this purpose, EndoH analysis was combined with *in vitro* galactosylation of the whole antibody performed as described (Raju et al., 2001). The aim was to distinguish between hybrid or complex oligosaccharides by making use of the fact that hybrid oligosaccharides can be galactosylated only at one terminal GlcNAc residue.

## Binding of Monomeric IgG1 Glycovariants to Natural Killer (NK) Cells and Fc<sub>γ</sub>RIIIa-Expressing CHO Cell Line

Human NK cells were isolated from freshly isolated peripheral blood mononuclear cells (PBMC) applying a negative selection enriching for CD16- and CD56-positive cells (MACS system, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The purity determined by CD56 expression was between 88% and 95%. Freshly isolated NK cells were incubated in PBS without calcium and magnesium ions ( $3 \times 10^5$  cells/mL) for 20 min at 37°C to remove NK cell-associated IgG. Cells were incubated at  $10^6$  cells/mL at different concentrations of anti-CD20 antibody (0, 0.1, 0.3, 1, 3, 10 μg/mL) in PBS, 0.1% BSA. After two washes with PBS, 0.1% BSA antibody, binding was detected by incubating with 1:200 FITC-conjugated F(ab')<sub>2</sub> goat anti-human, F(ab')<sub>2</sub> specific IgG (Jackson ImmunoResearch, West Grove, PA), and anti-human CD56-PE (BD Biosciences, Shields et al., 2002). The anti-Fc<sub>γ</sub>RIIIa 3G8 F(ab')<sub>2</sub> fragments (Anecll, Bayport, MN) were added at a concentration of 10 μg/mL to compete binding of antibody glycovariants (3 μg/mL). Fluorescence intensity was determined for CD56-positive cells on a FACSCalibur (BD Biosciences) and refers to the geometric mean measured for different antibody concentrations, from which the

geometric mean of cells incubated without primary antibody was subtracted.

CHO cells were transfected by electroporation (280 V, 950 μF, 0.4 cm) with an expression vector coding for the Fc<sub>γ</sub>RIIIa-Val158 α-chain and the γ-chain. Transfectants were selected by addition of 6 μg/mL puromycin and stable clones were analyzed by FACS using 10 μL FITC-conjugated anti-Fc<sub>γ</sub>RIII 3G8 monoclonal antibody (BD Biosciences) for  $10^6$  cells. Binding of IgG1 to Fc<sub>γ</sub>RIIIa-Val158-expressing CHO cells was performed analogously to the NK cell binding described above by omitting CD56-staining.

## Biological Activity of Anti-CD20 Monoclonal Antibody Glycovariants

### Antibody-Dependent Cellular Cytotoxicity (ADCC) Assay

CD20-positive Raji cells (DMEM, 10% FCS, 1% Glutamax, Invitrogen AG) were labeled with the fluorescent dye Calcein AM for 20 min, according to the manufacturer's instruction (Molecular Probes, Leiden, The Netherlands). Antibodies were serially diluted in AIM-V (Invitrogen AG) and incubated with the target cells for 10 min at room temperature prior to the addition of effector cells. PBMCs were prepared from a donor heterozygous for Fc<sub>γ</sub>RIIIa-Val/Phe158 and lacking Fc<sub>γ</sub>RIIc expression using Histopaque-1077 (Sigma-Aldrich, Buchs, Switzerland) following the manufacturer's instructions. PBMCs were added to the wells at an effector to target ratio of 25:1. After 4 h incubation at 37°C, the cells were spun down, washed twice with PBS without calcium and magnesium ions, and lysed by addition of 50 mM borate, 0.1% Triton X-100 solution. The content of the wells was subsequently transferred to a 96-well black flat-bottomed plate. Retention of the fluorescent dye by intact target cells was measured with a fluorometer (485 nm excitation, 520 nm emission, FLUOstar Optima, BMG Labtechnologies, Inc., Durham, NC). Specific lysis was calculated relative to the total lysis control, resulting from incubating the target cells with 1% Triton X-100. Percentage of specific antibody-mediated cytotoxicity was calculated as follows:

$$\% \text{ cytotoxicity} = ((\text{Fluorescence at concentration } x - \text{Fluorescence of spontaneous release}) / (\text{Fluorescence maximal release} - \text{Fluorescence spontaneous release})) \times 100$$

Each antibody dilution was analyzed in quadruplicate.

### Complement-Dependent Cytotoxicity (CDC) Assay

CD20 positive human B lymphoblastoid SKW 6.4 cells (DMEM, 10% FCS, 1% Glutamax, Invitrogen AG) were incubated with increasing concentrations of antibody for 10 min at room temperature prior to the addition of normal human serum (NHS, Fc<sub>γ</sub>RIIIa-Val158 and Fc<sub>γ</sub>RIIC homozygous donor), prepared from the blood of healthy

volunteers, as source of complement. The blood was allowed to coagulate for 1 h at room temperature and then centrifuged at 1,200g for 20 min. The serum was diluted threefold with AIM-V and added to the wells to obtain a final concentration of 20% NHS. The assay plates were incubated for 2 h at 37°C. Maximal release was determined by incubating the cells in the presence of 1% Triton X-100. Relative cytotoxicity was determined using the AlamarBlue™ Assay (Serotec, Inc., Düsseldorf, Germany). Fluorescence was monitored on a fluorometer (540 nm excitation, 590 nm emission, FLUOstar Optima, BMG Labtechnologies, Inc.). Each antibody concentration was measured in quadruplicate.

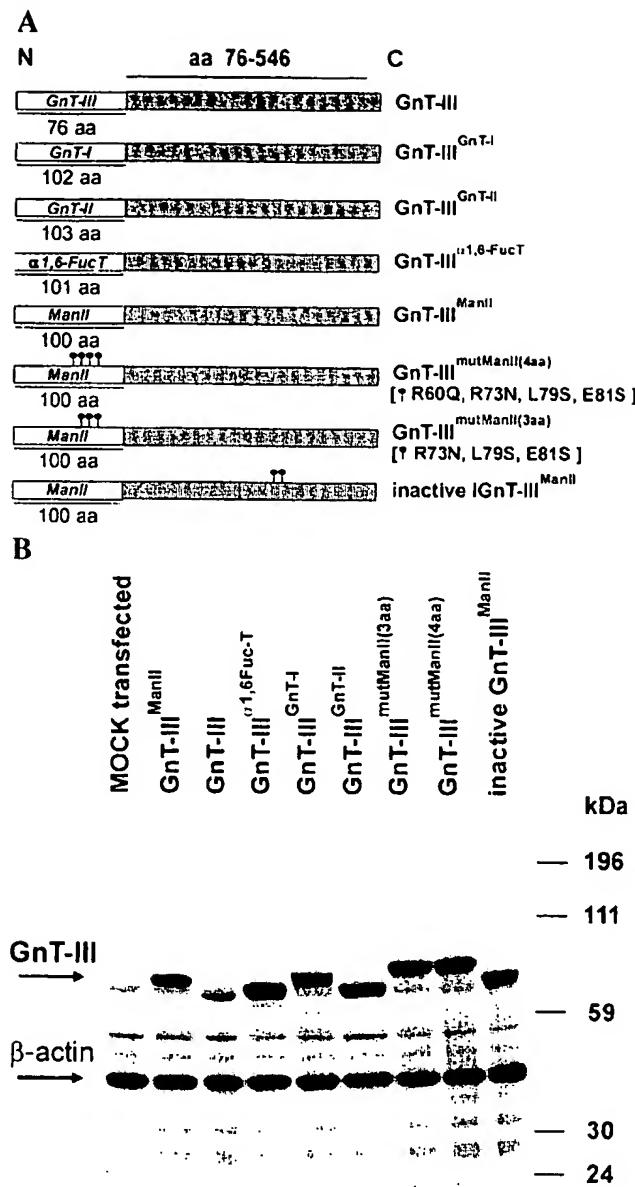
### Whole Blood B Cell Depletion Assay

Four hundred ninety microliters heparinized blood from a healthy Fc<sub>γ</sub>RIIA-Val158 and Fc<sub>γ</sub>RIIC negative donor, were incubated at 37°C with 10 µL PBS or 50-fold concentrated antibody glycovariants (final concentrations of 0.1, 1, 10, 100, 1,000 ng/mL). After 24 h 50 µL blood were stained with a mixture of anti-CD19-PE, anti-CD3-FITC, and anti-CD45-CyChrome (BD Biosciences) for 15 min at room temperature. Before analysis, 500 µL PBS containing 2% FCS and 5 mM EDTA were added to the tubes. The CD3-FITC and CD19-PE fluorescence of the blood samples were flowcytometrically analyzed by gating on all CD45-positive cells. B cell-depletion was determined by plotting the ratio of CD19-positive B cells to CD3-positive T cells. Each antibody concentration was analyzed in triplicate.

## RESULTS

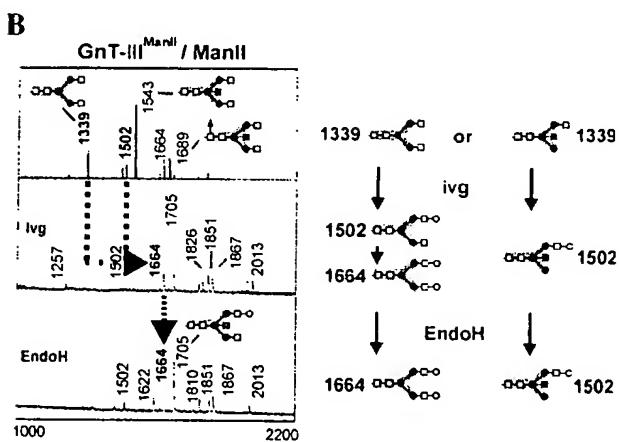
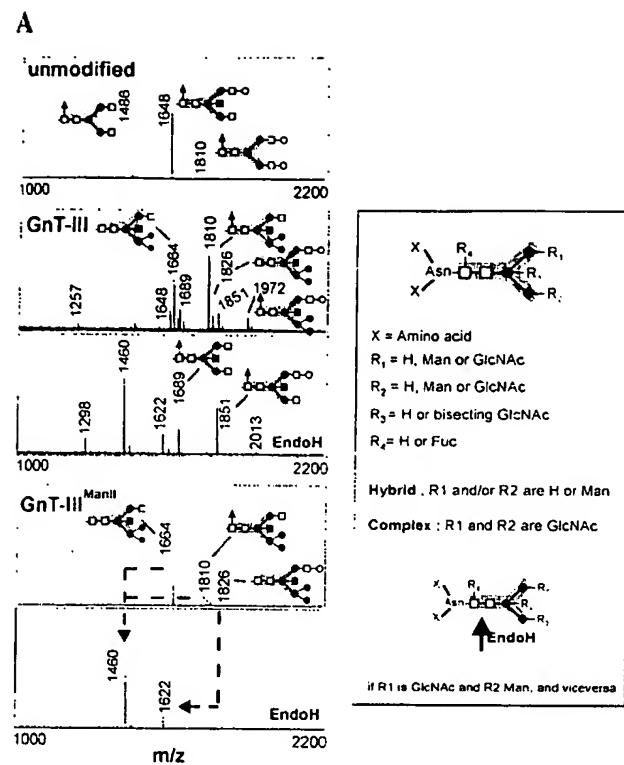
### Generation of Anti-CD20 Antibody Glycovariants Achieved by Co-Expression of GnT-III or Chimeric GnT-III Proteins

Modulation of antibody glycosylation was achieved by co-expression of the genes coding for antibody and chimeric GnT-III proteins in HEK293-EBNA cells. In the chimeric proteins, the localization domain of GnT-III was replaced with those of various other Golgi-resident enzymes of the N-linked glycosylation pathway (Fig. 1A). The oligosaccharide profiles obtained for the expressed antibodies indicate a significant impact of the enzyme localization on the outcome of the glycosylation process (Fig. 2A, Table I). The expression of GnT-III leads to antibodies with high proportions of bisected oligosaccharides, which can be fucosylated or non-fucosylated and of the complex or hybrid type. The assignment of these structures to the peaks was confirmed by digestion with EndoH, an enzyme digesting high mannose and most hybrid, but not complex oligosaccharides. Thus, peaks at *m/z* 1,664 and 1,810 shift to 1,460, and those at 1,826 and 1,972 to 1,622, confirming the increase in bisected, non-fucosylated hybrid (*m/z* 1,664 and 1,826) and their fucosylated versions (*m/z* 1,810 and 1,972) upon recombinant expression of GnT-III (Fig. 2A and B). The use of the localization domain of GnT-I,



**Figure 1.** A:  $\beta$ 1,4-N-acetylgalactosaminyltransferase III (GnT-III) chimeric proteins. GnT-III with different localization domains were constructed by fusing the catalytic domain of GnT-III (gray) to the localization domain CTS (white), consisting of cytoplasmic tail, transmembrane domain, and stem region of other Golgi-resident enzymes. The length of the respective regions is indicated. In the last three constructs single amino acid substitutions are indicated. B: Western blot detection of GnT-III. Fifteen micrograms of cell lysates were separated on a 4–12% NU-PAGE gel (Invitrogen AG, Basel, Switzerland) and electroblotted to nitrocellulose membrane. Detection of GnT-III was performed via its C-terminal c-myc tag, while  $\beta$ -actin detection served as an internal control for the amount of loaded extracts (Abeam Ltd, Cambridge, UK).

$\alpha 1,6\text{-FucT}$ , GnT-II, or ManII, instead of that of GnT-III results in a further increase in the proportion of bisected non-fucosylated hybrid oligosaccharides (mainly *m/z* 1,664) linked to the secreted antibody. Among these chimeric proteins, GnT-III fused to the localization domain of ManII (GnT-III<sup>ManII</sup>) leads to the highest content of these carbohydrates (Table I, Fig. 2A).



**Figure 2.** MALDI-TOF-MS spectra of neutral oligosaccharides from recombinant anti-CD20 IgG1 glycovariants. The antibodies were produced in HEK293-EBNA cells engineered for expression of various forms of GnT-III (see Fig. 1a). The  $m/z$  value corresponds to the sodium-associated oligosaccharide ion. A: MALDI-TOF-MS spectra of neutral oligosaccharides released from unmodified, GnT-III-modified, and GnT-III<sup>ManII</sup>-modified antibodies. Endoglycosidase H digestion (EndoH) was performed to confirm the increase in bisected non-fucosylated hybrid ( $m/z$  1,664 and 1,826) and their fucosylated versions ( $m/z$  1,810 and 1,972) upon expression of recombinant GnT-III. EndoH digestion of the released oligosaccharides causes a shift for peaks at  $m/z$  1,664 and 1,810 (to  $m/z$  1,460) and of peaks at  $m/z$  1,826 and 1,972 (to  $m/z$  1,622). Complex oligosaccharides, as peaks at  $m/z$  1,689 and 1,851 are not digested by EndoH. B: MALDI-TOF-MS spectra of neutral oligosaccharides released from antibodies engineered by co-expression with GnT-III<sup>ManII</sup> and ManII genes. In vitro galactosylation ("ivg") was performed to refine the oligosaccharide analysis and confirm that the peak  $m/z$  1,339 corresponds to complex non-fucosylated oligosaccharides.

**Table 1.** Oligosaccharide distributions (relative percentages) of anti-CD20 monoclonal antibody glycoforms.

	Unmodified	GnT-III	GnT-III <sup>GnT-III</sup>	GnT-III <sup>2,3,6-<i>GalT-II</i></sup>	GnT-III <sup>2,3,6-<i>GalT-II</i>,<i>ManII</i></sup>	GnT-III <sup>ManII</sup>	GnT-III <sup>2,3,6-<i>GalT-II</i>,<i>ManII</i></sup>	iGnT-III <sup>ManII</sup>	GnT-III <sup>ManII</sup>	GnT-III <sup>ManII</sup> /ManII
High mannose	1	2	21	6	3	—	3	1	—	—
Hybrid	v3	—	—	—	—	—	—	6	—	—
Non-bisected non-fucosylated	—	—	—	—	—	—	—	—	—	—
Non-bisected non-fucosylated	—	—	29	15	27	10	38	—	—	—
Bisected non-fucosylated	—	—	22	64	66	90	56	62	2	18
Bisected non-fucosylated	95	5	—	6	4	—	3	2	83	8
Complex	—	—	—	—	—	—	—	—	—	—
Non-bisected fucosylated	—	—	42	—	—	—	—	—	8	13
Non-bisected fucosylated	—	—	—	—	—	—	—	—	15	26
Bisected fucosylated	—	—	—	—	—	—	—	—	15	49
Bisected fucosylated	—	—	—	—	—	—	—	—	43	49
Total bisected	—	93	79	87	93	100	94	95	2	77
Total non-fucosylated	—	22	64	52	66	90	56	62	10	76
Total non-fucosylated, complex	1	—	—	—	—	—	—	—	8	58
Total complex	96	47	—	7	4	—	3	2	91	81
										96

Designation according to Figure 1a.

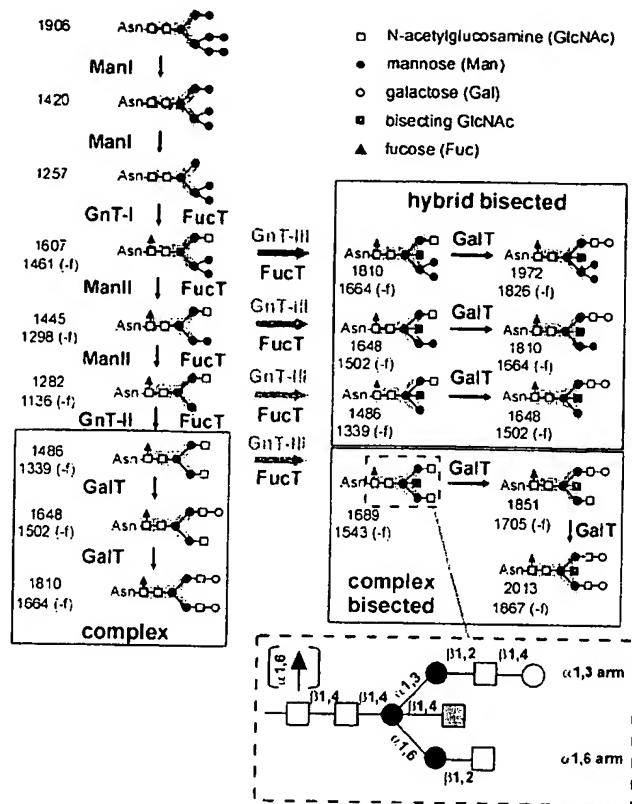
Unmodified antibody is produced by transient expression in HEK293-EBNA cells by omitting the expression vectors coding for the carbohydrate-modifying enzymes. The percentages obtained are relative to the total sum of the intensity of each peak. For hybrid bisected oligosaccharides, additional EndoH digestion confirmed their assignment.

To assess if the localization domain of ManII itself and not the enzymatic activity of the catalytic domain of GnT-III accounts for any of the oligosaccharide modifications, a catalytically inactive iGnT-III<sup>ManII</sup> chimeric protein was prepared by replacing residues Asp321 and Asp323 of GnT-III by alanine residues (Ihara et al., 2002) (Fig. 1A). The spectrum of the glycans modified by the inactive chimeric protein indicates that the catalytic domain of GnT-III is mainly responsible for the oligosaccharide profile of the GnT-III<sup>ManII</sup>-modified antibody. The reason for the minor increase in the fraction of complex non-fucosylated oligosaccharides needs further investigation (Table I). To ascertain if a different expression level of the enzymes may account for the differences in the oligosaccharide patterns, the expression of GnT-III was quantified by Western blot analysis via a C-terminal c-myc tag (Fig. 1B). Both GnT-III<sup>ManII</sup> and GnT-III showed a slightly reduced expression compared to those of the other GnT-III chimeric proteins.

We also evaluated the hypothesis that the existence of relatively well organized functional glycosylation reaction subcompartments within the medial and trans Golgi cisternae may account for the glycosylation profiles derived from the chimeric GnT-III proteins. Given that pairs of charged amino acid residues in the stem regions of GnT-I and ManII have been postulated as critical for oligomer formation between enzymes (Nilsson et al., 1996), it was investigated if such a pairing could account for the GnT-III<sup>ManII</sup>-derived antibody glycosylation profile. Therefore the amino acid mutations R73N, L79S, and E81S (GnT-III<sup>mutManII(3aa)</sup>) or R60Q, R73N, L79S, and E81S (GnT-III<sup>mutManII(4aa)</sup>) were introduced into the stem region of ManII (Fig. 1A). Both mutants were expressed in similar amounts (Fig. 1B) and yielded antibody glycovariants featuring substantially reduced proportions of bisected non-fucosylated oligosaccharides compared to the non-mutated GnT-III<sup>ManII</sup> (Table I).

Either GnT-III<sup>ManII</sup> or GnT-III was co-expressed with ManII to shift the biosynthetic pathway from hybrid to complex bisected oligosaccharides (Fig. 2B). The expression of both enzyme combinations lead to the generation of antibodies characterized by high proportions of complex type glycans lacking core-fucosylation, with the majority being bisected (Table I). In vitro galactosylation analysis confirmed that peaks at *m/z* 1,339 and 1,502, which were not digested by EndoH, could be assigned to non-fucosylated complex glycan structures, with only minor contribution of bisected non-fucosylated hybrid structures (Fig. 2, Table I). GnT-III<sup>ManII</sup> was also co-expressed with GnT-II, an enzyme that similarly to ManII directs the glycosylation pathway toward the formation of complex type glycans. This led to the accumulation of high mannose structures and a low proportion of bisected non-fucosylated oligosaccharides (data not shown), and this enzyme combination was not investigated further.

Two glyco-engineered antibodies, namely those produced either by transient co-expression with GnT-III<sup>ManII</sup> (termed "Glyco-1," bearing mainly hybrid non-fucosylated bisected glycans) or with GnT-III<sup>ManII</sup> and ManII ("Glyco-2," bearing

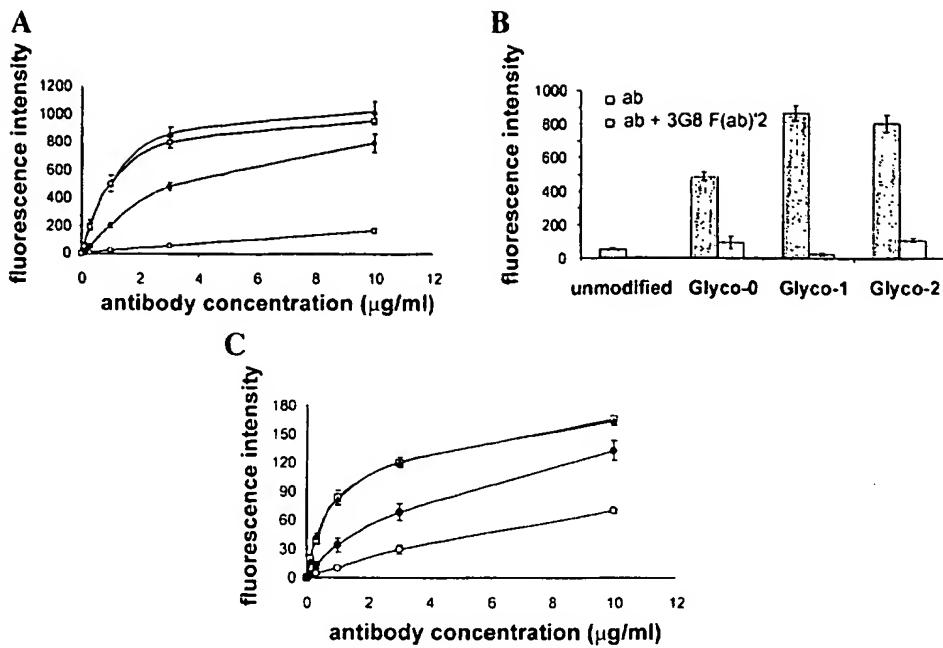


**Figure 3.** *N*-linked oligosaccharide biosynthetic pathway leading to complex or hybrid structures bearing a bisecting GlcNAc. The mass to charge (*m/z*) value of the sodium-associated oligosaccharide ion obtained by MALDI/TOF-MS analysis is indicated next to the corresponding oligosaccharide structure. The shaded monosaccharides belong to the core of the oligosaccharide, shared by all naturally occurring *N*-linked glycoforms; the presence of the other sugars is variable. A bisecting *N*-acetylglucosamine (GlcNAc; gray) can be  $\beta$ 1,4 linked to the core mannose by the enzyme GnT-III, as long as the oligosaccharide has been previously modified by GnT-I.  $\alpha$ 1,6-fucosyltransferase ( $\alpha$ 1,6-FucT) catalyses the addition of a fucose  $\alpha$ 1,6-linked to the GlcNAc residue attached to Asn, to any oligosaccharides that have not been modified by GnT-III or GaIT. Complex and hybrid glycans structures are defined by the structure of the  $\alpha$ 1,6 arm. ManI, mannosidase I; GnT-I,  $\beta$ 1,2-*N*-acetylglucosaminyltransferase I; ManII, Golgi  $\alpha$ -mannosidase II; GnT-II,  $\beta$ 1,2-*N*-acetylglucosaminyltransferase II; GaIT,  $\beta$ 1,4-galactosyltransferase (GaIT); GnT-III,  $\beta$ 1,4-*N*-acetylglucosaminyltransferase III;  $\alpha$ 1,6-FucT, core  $\alpha$ 1,6-fucosyltransferase.

mainly complex non-fucosylated bisected glycans) were examined for their affinity for Fc $\gamma$ RIIIa and compared either to the unmodified antibody or to an antibody glycovariant produced under the same conditions by transient co-expression of GnT-III ("Glyco-0") (Fig. 3).

### Fc $\gamma$ RIIIa Binding of Glyco-0, Glyco-1, and Glyco-2 Anti-CD20 Monoclonal Antibodies

Binding of glyco-engineered antibodies to Fc $\gamma$ RIIIa was evaluated on peripheral human natural killer (NK) cells, which are known to be important mediators of ADCC, and to constitutively express Fc $\gamma$ RIIIa. Binding to NK cells was performed by incubating the antibody glycovariants with freshly isolated NK cells from a donor who was genotyped as heterozygous for Fc $\gamma$ RIIIA-Val/Phe158 (Koene et al., 1997).



**Figure 4.** Fc<sub>γ</sub>RIIIa binding of monomeric anti-CD20 glycovariant antibodies. Binding to purified NK cells from a donor heterozygous for Fc<sub>γ</sub>RIIIA-Val/Phe 158 and negative for Fc<sub>γ</sub>RIIC was evaluated. **A:** Binding of antibodies at concentrations ranging from 0.1 to 10 µg/mL. **B:** Antibody binding (3 µg/mL) to Fc<sub>γ</sub>RIIIA was competed by addition of blocking anti-Fc<sub>γ</sub>RIIIa 3G8 F(ab')<sub>2</sub> fragments (10 µg/mL). **C:** Binding to a CHO cell line stably expressing human Fc<sub>γ</sub>RIIIa-Val 158  $\alpha$ -chain and  $\gamma$ -chain was evaluated to confirm the dependence of antibody binding to Fc<sub>γ</sub>RIIIa. All assays were performed in quadruplicate. ○, unmodified; ●, Glyco-0; ▲, Glyco-1; and □, Glyco-2 antibodies.

Both Glyco-1 and Glyco-2 bind with a considerably higher affinity to NK cells than unmodified antibody (Fig. 4A). Under the transient gene expression levels of this study, GnT-III co-expression leads to an antibody (Glyco-0) with a lower level of bisected non-fucosylated oligosaccharides with intermediate Fc<sub>γ</sub>RIIIa binding affinity. Antibody binding to NK cells occurred exclusively via Fc<sub>γ</sub>RIIIa as it could be outcompeted by the addition of blocking anti-Fc<sub>γ</sub>RIIIa F(ab')<sub>2</sub> fragments (Fig. 4B). Similar results were obtained using a recombinant CHO cell line stably expressing the Fc<sub>γ</sub>RIIIa-Val158  $\alpha$ -chain receptor (Fig. 4C).

#### Biological Activity of Glyco-1 and Glyco-2 Anti-CD20 Monoclonal Antibodies

In a next step we investigated whether increased Fc<sub>γ</sub>RIIIa binding correlates with an improvement in the biological activity of the glyco-engineered antibodies, which are characterized by bisected non-fucosylated oligosaccharides. Both glycovariants mediate an enhanced ADCC against CD20-positive Raji cells, independently of the Fc-linked glycans being of complex or hybrid type (Fig. 5A).

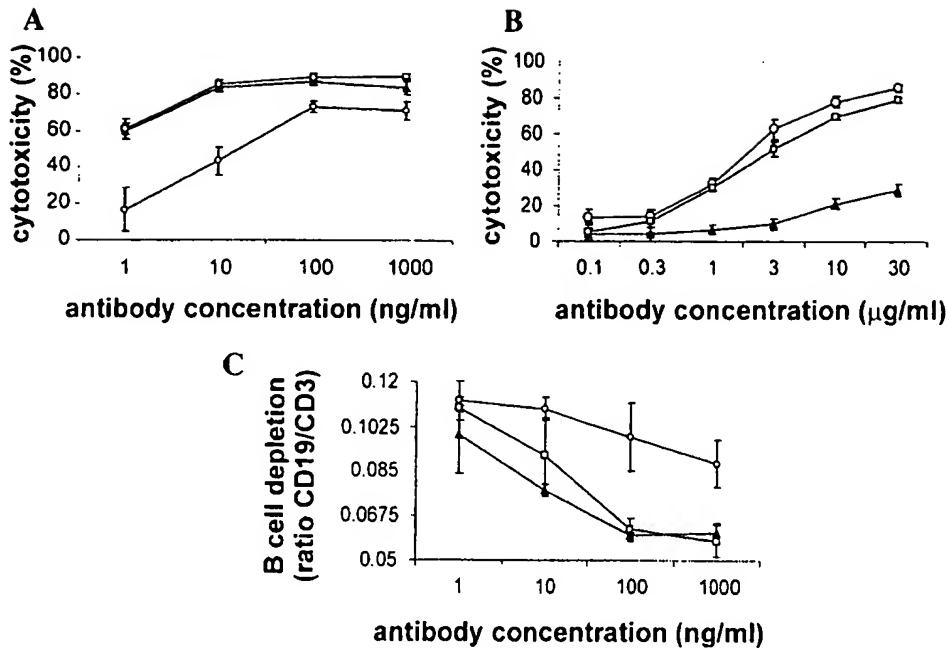
Glyco-1 and Glyco-2 were also evaluated for complement-dependent cytotoxicity (CDC) against CD20-positive tumor cells SKW6.4 in the presence of human serum (Fig. 5B). Glyco-2, bearing complex oligosaccharides, performs similarly to the unmodified antibody in CDC. For Glyco-1, on the contrary, a reduction in CDC is observed, correlating with high proportion of hybrid oligosaccharides that are characteristic for this glycovariant.

To assess whether both ADCC and CDC can contribute to the elimination of target cells, we evaluated these glyco-engineered antibodies for B cell depletion in a whole blood assay (Fig. 5C). Glyco-1 and Glyco-2 anti-CD20 variants were over 100-fold more potent than unmodified anti-CD20 in depleting B cells, while no significant difference could be observed between the two glycovariants, indicating a minor contribution of CDC.

## DISCUSSION

#### Chimeric GnT-III Proteins

GnT-III is an ideal enzyme to manipulate the *N*-glycosylation of expressed proteins (glyco-engineering), as it exerts a large degree of control over the glycosylation process by blocking the action of  $\alpha$ 1,6-FucT, ManII, and GnT-II (Schachter, 1986; Umaña et al., 1999). While natural human IgG in serum contains low percentages of bisected oligosaccharides in the Fc region (Wormald et al., 1997), up to 40% bisected glycans were observed for antibodies produced in YB2/0 rat myeloma cell line (Lifely et al., 1995). In the present study the impact of the localization domain of GnT-III on the antibody glycosylation profile was evaluated. We obtained antibodies with engineered carbohydrate moieties by co-expression of chimeric GnT-III proteins, composed of the catalytic domain of GnT-III fused to the localization domain of GnT-I,  $\alpha$ 1,6-FucT, GnT-II, or ManII (Fig. 1A). As for the GnT-III-derived antibody, the resulting glycovariants feature a bisecting *N*-acetylglucosamine (GlcNAc) residue on



**Figure 5.** Biological activity assays of anti-CD20 antibody glycovariants. **A:** ADCC using PBMCs (Fc<sup>+</sup>RIIIA-Val/Phe158, Fc<sup>+</sup>RIIC negative donor) as effectors and human lymphoma Raji cells as targets. **B:** CDC against B lymphoblastoid SKW6.4 cells in the presence of human serum as a source of complement. **C:** B-cell depletion in whole blood (Fc<sup>+</sup>RIIIA-Val/Phe158, Fc<sup>+</sup>RIIC negative donor), which was calculated from the ratio of CD19-positive B cells to CD3-positive T cells as measured by FACS analysis. ○, unmodified; ▲, Glyco-1; and □, Glyco-2 antibodies.

almost all oligosaccharide structures, indicating that all chimeric GnT-III proteins were active (Table I, Fig. 2A). Relative to GnT-III-modified antibody, use of chimeric GnT-III led to an increase in the fractions of bisected non-fucosylated, and of bisected hybrid oligosaccharides, indicating a more efficient competition of the chimeric GnT-III against  $\alpha$ 1,6-FucT and ManII (Fig. 2A).

Among the chimeric GnT-III proteins, GnT-III<sup>ManII</sup> is the most efficient in the competition against the above-mentioned enzymes, leading to the antibody glycovariant with the highest proportion of bisected non-fucosylated hybrid oligosaccharides. Since the expression level of GnT-III<sup>ManII</sup> was not higher than for the other chimeric proteins or GnT-III, we propose that the higher efficiency of GnT-III<sup>ManII</sup> results either from a different distribution in Golgi compartments or from a different functional organization of enzymes within a compartment. This allows GnT-III to act immediately after GnT-I in the biosynthetic process, leading to higher levels of bisected, non-fucosylated hybrid oligosaccharides (Table I) relative to the other chimeric GnT-III proteins.

#### Influence of the ManII Stem Region Residues on the Glycosylation Pattern

The higher efficiency of the chimeric protein GnT-III<sup>GnT-I</sup>, compared to the unmodified GnT-III, for the synthesis of bisected hybrid and bisected non-fucosylated oligosaccharides can be explained by an earlier Golgi distribution, in the *cis*-to-*trans* direction of glycoprotein substrate transport, of GnT-I relative to GnT-III. The fine Golgi distributions of

GnT-I and ManII have been determined previously by quantitative immunoelectron microscopy (Rabouille et al., 1995). Both enzymes co-distribute along the Golgi, being localized mainly in the medial and *trans* cisternae (Dunphy et al., 1985; Rabouille et al., 1995; Velasco et al., 1993). The spatial distributions of  $\alpha$ 1,6-FucT and GnT-II have not yet been determined quantitatively, but rat GnT-III has been found predominantly in the *trans* Golgi cisternae (Umaña, 1998). This, however, does not explain why the chimeric GnT-III<sup>ManII</sup> is significantly more efficient than GnT-III<sup>GnT-I</sup> at synthesizing bisected, hybrid, and bisected, non-fucosylated oligosaccharides, since both GnT-I and ManII have identical spatial distributions along the Golgi subcompartments. Additionally, the slightly lower expression level of GnT-III<sup>ManII</sup> compared to those of the other chimeric GnT-III proteins, indicates that the antibody glycoprofile is not a result of an increased enzyme expression level (Fig. 1B), but a consequence of a more efficient processing of GnT-I-modified oligosaccharides, denoted here as “functional pairing” of GnT-III<sup>ManII</sup> with GnT-I.

To assess if this functional pairing relies on a physical interaction between the two enzymes, we evaluated GnT-III<sup>ManII</sup> mutants with amino acid substitutions in the localization domain of ManII which were reported to be critical determinants for the formation of hetero-oligomers of GnT-I and ManII (Nilsson et al., 1996). Although it has been suggested that these residues are not essential for incorporation into high molecular weight complexes of Golgi enzymes or even for Golgi localization (Opat et al., 2000), it is possible that they are involved in a finer pairing of the catalytic

domains during oligosaccharide biosynthesis. We observed a significant reduction in bisected non-fucosylated hybrid oligosaccharides with these mutants (Table I). Both GnT-III<sup>mutManII</sup> mutants were expressed at comparable levels as GnT-III<sup>ManII</sup>, excluding the expression level of the latter as being responsible for higher proportions of bisected non-fucosylated glycans (Fig. 1B). However, the exchanged residues do not seem to be the sole determinant of the resulting oligosaccharide product distribution, suggesting either additional contributions of the rest of the stem or catalytic regions to functional enzyme pairing, or an enrichment of these enzymes in subcompartments caused by different mechanisms. Evidence for pairing may possibly be obtained via co-immunoprecipitation and electron microscopy experiments. Another possible explanation may be a conformational change caused by the modified stem region, which may lead to a catalytic domain with increased activity. Our data suggest that by virtue of the ManII localization domain, a physical and/or a functional pairing takes place between the catalytic domains of the endogenous GnT-I and the recombinant GnT-III<sup>ManII</sup> chimeric protein.

### Co-Expression of GnT-III<sup>ManII</sup> and ManII

With the described approach, we are able to modulate the glycosylation pattern of antibodies from fucosylated complex glycans to bisected non-fucosylated hybrid oligosaccharides by overexpressing GnT-III<sup>ManII</sup>. The co-expression of GnT-III<sup>ManII</sup> and ManII, or of GnT-III and ManII led to the formation of bisected non-fucosylated glycans of the complex type. ManII overexpression redirects the biosynthetic pathway causing the product shift from hybrid to complex carbohydrates (Fig. 2B, Table I). Although equally high levels of bisected non-fucosylated complex oligosaccharides can be synthesized by high level expression of GnT-III, the results presented here show that GnT-III<sup>ManII</sup> is more efficient at adding a bisecting GlcNAc residue to the GnT-I-processed oligosaccharides prior to the reactions catalyzed by ManII, GnT-II, and  $\alpha$ 1,6-FucT.

Similarly to ManII, GnT-II was co-expressed with GnT-III<sup>ManII</sup> with the intent of forming complex type glycans linked to the antibody. The resulting glycovariant had lower proportions of bisected non-fucosylated (37%) and complex (22%) glycans compared to the GnT-III<sup>ManII</sup>/ManII-derived antibody (data not shown). Moreover, a significant fraction of high mannose oligosaccharides characterized this glycovariant, suggesting an influence of the overexpression of GnT-II on the maturation process of high mannose glycans. Under these conditions, the enzyme seems to inhibit the GnT-I-mediated reaction (Table I) by unknown mechanisms. A similar phenomenon was reported for overexpression of  $\beta$ 1,4-GaIT, which led to an enrichment of high mannose oligosaccharides on recombinantly co-expressed IFN- $\gamma$  (Fukuta et al., 2001). Although the cellular localization of GnT-II is yet to be discovered, high molecular weight complexes between GnT-I and GnT-II have been found in

Golgi extracts of mammalian cells (Opat et al., 2000). The formation of such complexes might be disturbed by GnT-II overexpression.

We could show that the formation of a desired carbohydrate profile can be achieved by the combination and overexpression of enzymes, although not all the potential enzymes are suitable for this purpose, indicating that the glycosylation process is governed by a well-balanced system of enzymes that needs further elucidation.

### Biological Activity of Glyco-Engineered Antibodies

A high affinity to Fc $\gamma$ RIIIa is important for ADCC, which is mediated by unconjugated therapeutic antibodies in humans. This was deduced from pioneering pharmacogenomic studies evaluating the impact of the Fc $\gamma$ RIIIA polymorphisms on the activity of rituximab in lymphoma patients (Cartron et al., 2002). In that study, the objective response rates at 2/12 months were 100/90% for homozygous Fc $\gamma$ RIIIA-Val158 and 67/51% for Fc $\gamma$ RIIIA-Phe158 carriers, respectively. The superior response of the former seems to be the result of a significantly increased binding of the antibody to Fc $\gamma$ RIIIa-Val158 compared to Fc $\gamma$ RIIIa-Phe158 (Koene et al., 1997).

Glyco-1, featuring mainly bisected non-fucosylated hybrid glycans, and Glyco-2, bearing mainly bisected non-fucosylated complex carbohydrates, were examined for their binding to Fc $\gamma$ RIIIa and their reactivity in cytotoxicity assays. Both Glyco-1 and Glyco-2 have an increased affinity for Fc $\gamma$ RIIIa, which correlates with high proportions of bisected non-fucosylated oligosaccharides but seems independent of the glycans being of the hybrid or complex type. It has been reported that the absence of core fucose is responsible for an increased affinity to Fc $\gamma$ RIIIa (Shields et al., 2002). Moreover, the absence of core fucose but not the presence of galactose or bisecting GlcNAc was reported to be responsible for increased ADCC under the tested conditions (Shinkawa et al., 2003).

Both Glyco-1 and Glyco-2 mediate an increased ADCC to a similar extent compared to the unmodified antibody, but feature a different reactivity in CDC assays. While Glyco-2 acts similarly to the unmodified antibody, Glyco-1 displays a reduced CDC, suggesting a significant influence of the glycan type (complex vs. hybrid). The main difference between Glyco-1 and Glyco-2 is the structure of their carbohydrate  $\alpha$ 1,6-arm. In contrast to Glyco-1, carrying hybrid glycans with mannose residues  $\alpha$ 1,3- and  $\alpha$ 1,6-linked to the  $\alpha$ -6 arm. Glyco-2 and unmodified antibodies have mainly  $\beta$ 1,2-linked GlcNAc residues at this position (complex glycans), which may be followed by galactose. This carbohydrate arm is in close contact with the IgG-C $\gamma$ 2 domain polypeptide and may therefore influence domain conformations required for binding to C1q (Duncan and Winter, 1988; Huber et al., 1976; Idusogie et al., 2000). An even larger reduction in CDC was reported for antibodies featuring only high mannose oligosaccharides (Wright and Morrison, 1994).

The glyco-engineered antibodies also performed better than their unmodified counterparts in the depletion of B-cells in a whole blood assay, where both ADCC and CDC contribute to the elimination of target cells. The reduction in CDC activity, observed for Glyco-I, does not seem to affect our model of B-cell depletion in whole blood, suggesting that ADCC is the predominant mechanism in this assay. Moreover as first-dose-related side effects in vivo have been recently attributed to complement activation (van der Kolk et al., 2001; Winkler et al., 1999), Glyco-I may provide a tool to prevent these problems.

We could therefore demonstrate that apart from modulating glycosyltransferase expression levels, engineering of Golgi localization domains can also be exploited for the production of tailored glyco-engineered therapeutic antibodies with unique combinations of biological activities.

We would like to thank René Fischer (Laboratory of Organic Chemistry, ETH Zürich, Switzerland) for providing the HEK293-EBNA cell line and Patrik Buholzer, Christian Gerdes, Samuel Haldane, Saskia Karg, Julia Maria Martinez, Peter Sondermann, and Manuel Späni for assistance and useful comments on this manuscript.

## References

- Carter P. 2001. Improving the efficacy of antibody-based cancer therapies. *Nat Rev Cancer* 1:118–129.
- Cartron G, Dacheux L, Salles G, Solal-Celigny P, Bardos P, Colombat P, Watier H. 2002. Therapeutic activity of humanized anti-CD20 monoclonal antibody and polymorphism in IgG Fc receptor FcgammaRIIIa gene. *Blood* 99:754–758.
- Dall'Ozzo S, Turtas S, Pautraud G, Cartron G, Colombat P, Bardos P, Watier H, Thibault G. 2004. Rituximab-dependent cytotoxicity by natural killer cells: Influence of FCGR3A polymorphism on the concentration-effect relationship. *Cancer Res* 64:4664–4669.
- Duncan AR, Winter G. 1988. The binding site for Clq on IgG. *Nature* 332(6166):738–740.
- Dunphy WG, Brands R, Rothman JE. 1985. Attachment of terminal N-acetylglucosamine to asparagine-linked oligosaccharides occurs in central cisternae of the Golgi stack. *Cell* 40(2):463–472.
- Fukuta K, Abe R, Yokomatsu T, Minowa MT, Takeuchi M, Asanagi M, Makino T. 2001. The widespread effect of beta 1,4-galactosyltransferase on N-glycan processing. *Arch Biochem Biophys* 392(1):79–86.
- Huber R, Deisenhofer J, Colman PM, Matsushima M, Palm W. 1976. Crystallographic structure studies of an IgG molecule and an Fc fragment. *Nature* 264(5585):415–420.
- Idusogie EE, Presta LG, Gazzano-Santoro H, Totpal K, Wong PY, Ultsch M, Meng YG, Mulkerin MG. 2000. Mapping of the Clq binding site on rituxan, a chimeric antibody with a human IgG1 Fc. *J Immunol* 164(8):4178–4184.
- Ibara H, Ikeda Y, Koyota S, Endo T, Honke K, Taniguchi N. 2002. A catalytically inactive beta 1,4-N-acetylglucosaminyltransferase III (GnT-III) behaves as a dominant negative GnT-III inhibitor. *Eur J Biochem* 269:193–201.
- Jeffries R, Lund J, Pound JD. 1998. IgG-Fc-mediated effector functions: Molecular definition of interaction sites for effector ligands and the role of glycosylation. *Immunol Rev* 163:59–76.
- Jordan M, Schallhorn A, Wurm FM. 1996. Transfecting mammalian cells: Optimization of critical parameters affecting calcium-phosphate precipitate formation. *Nucleic Acids Res* 24:596–601.
- Kobayashi N, Soderlind E, Borrebaeck CA. 1997. Analysis of assembly of synthetic antibody fragments: Expression of functional scFv with predefined specificity. *Biotechniques* 23:500–503.
- Koene HR, Kleijer M, Algra J, Roos D, von dem Borne AE, de Haas M. 1997. Fc gammaRIIIa-158V/F polymorphism influences the binding of IgG by natural killer cell Fc gammaRIIIa, independently of the Fc gammaRIIIa-48L/R/H phenotype. *Blood* 90:1109–1114.
- Lifely MR, Hale C, Boyce S, Keen MJ, Phillips J. 1995. Glycosylation and biological activity of CAMPATH-1H expressed in different cell lines and grown under different culture conditions. *J Glycobiol* 5:813–822.
- Metes D, Gamotto AA, Nellis J, Ruscin A, Stewart-Akers AM, Morel PA, Rao AS. 2001. Identification of the CD32/FcgammaRIIc-Q13/STP13 polymorphism using an allele-specific restriction enzyme digestion assay. *J Immunol Methods* 258:85–95.
- Nilsson T, Rabouille C, Hui N, Watson R, Warren G. 1996. The role of the membrane-spanning domain and stalk region of N-acetylglucosaminyltransferase I in retention, kin recognition and structural maintenance of the Golgi apparatus in HeLa cells. *J Cell Sci* 109(Pt 7):1795–1799.
- Opat AS, Houghton F, Gleeson PA. 2000. Medial Golgi but not late Golgi glycosyltransferases exist as high molecular weight complexes. Role of luminal domain in complex formation and localization. *J Biol Chem* 275:11836–11845.
- Papac DL, Briggs JB, Chin ET, Jones AJ. 1998. A high-throughput microscale method to release N-linked oligosaccharides from glycoproteins for matrix-assisted laser desorption/ionization time-of-flight mass spectrometric analysis. *Glycobiology* 8:445–454.
- Rabouille C, Hui N, Hunte F, Kieckbusch R, Berger EG, Warren G, Nilsson T. 1995. Mapping the distribution of Golgi enzymes involved in the construction of complex oligosaccharides. *J Cell Sci* 108(Pt 4):1617–1627.
- Raju TS, Briggs JB, Chamow SM, Winkler ME, Jones AJ. 2001. Glycoengineering of therapeutic glycoproteins: In vitro galactosylation and sialylation of glycoproteins with terminal N-acetylglucosamine and galactose residues. *Biochemistry* 40:8868–8876.
- Reff ME, Carner K, Chambers KS, Chinn PC, Leonard JE, Raab R, Newman RA, Hanna N, Anderson DR. 1994. Depletion of B cells in vivo by a chimeric mouse human monoclonal antibody to CD20. *Blood* 83:435–445.
- Schachter H. 1986. Biosynthetic controls that determine the branching and microheterogeneity of protein-bound oligosaccharides. *Biochem Cell Biol* 64:163–181.
- Shields RL, Namecuk AK, Hong K, Meng YG, Rue J, Briggs J, Xie D, Lai J, Stadlen A, Li B, Fox JA, Presta LG. 2001. High resolution mapping of the binding site on human IgG1 for Fc gamma RI, Fc gamma RII, Fc gamma RIII, and FcRn and design of IgG1 variants with improved binding to the Fc gamma R. *J Biol Chem* 276(9):6591–6604.
- Shields RL, Lai J, Keck R, O'Connell LY, Hong K, Meng YG, Weikert SH, Presta LG. 2002. Lack of fucose on human IgG1 N-linked oligosaccharide improves binding to human Fcgamma RIII and antibody-dependent cellular toxicity. *J Biol Chem* 277:26733–26740.
- Shinkawa T, Nakamura K, Yamane N, Shoji-Hosaka E, Kanda Y, Sakurada M, Uchida K, Anazawa H, Satoh M, Yamasaki M, Hanai N, Shitara K. 2003. The absence of fucose but not the presence of galactose or bisecting N-acetylglucosamine of human IgG1 complex-type oligosaccharides shows the critical role of enhancing antibody-dependent cellular cytotoxicity. *J Biol Chem* 278:3466–3473.
- Tao MH, Morrison SL. 1989. Studies of aglycosylated chimeric mouse-human IgG. Role of carbohydrate in the structure and effector functions mediated by the human IgG constant region. *J Immunol* 143:2595–2601.
- Umaña P. 1998. Engineering of protein glycosylation in Chinese hamster ovary cells. Pasadena, CA: California Institute of Technology. 155p.
- Umaña P, Jean-Mairet J, Moudry R, Amstutz H, Bailey JE. 1999. Engineered glycoforms of an antineuroblastoma IgG1 with optimized antibody-dependent cellular cytotoxic activity. *Nat Biotechnol* 17:176–180.

- van der Kolk LE, Grillo-Lopez AJ, Baars JW, Hack CE, van Oers MH. 2001. Complement activation plays a key role in the side-effects of rituximab treatment. *Br J Haematol* 115:807–811.
- Velasco A, Hendricks L, Morensten KW, Tulsiani DR, Touster O, Farquhar MG. 1993. Cell type-dependent variations in the subcellular distribution of alpha-mannosidase I and II. *J Cell Biol* 122(1):39–51.
- Winkler U, Jensen M, Manzke O, Schulz H, Diehl V, Engert A. 1999. Cytokine-release syndrome in patients with B-cell chronic lymphocytic leukemia and high lymphocyte counts after treatment with an anti-CD20 monoclonal antibody (rituximab, IDEC-C2B8). *Blood* 94:2217–2224.
- Wormald MR, Rudd PM, Harvey DJ, Chang SC, Scragg IG, Dwek RA. 1997. Variations in oligosaccharide-protein interactions in immunoglobulin G determine the site-specific glycosylation profiles and modulate the dynamic motion of the Fc oligosaccharides. *Biochemistry* 36(6):1370–1380.
- Wright A, Morrison SL. 1994. Effect of altered CH2-associated carbohydrate structure on the functional properties and in vivo fate of chimeric mouse-human immunoglobulin G1. *J Exp Med* 180(3):1087–1096.
- Wright A, Morrison SL. 1997. Effect of glycosylation on antibody function: Implications for genetic engineering. *Trends Biotechnol* 15:26–32.

# Biotechnology — and — Bioengineering

Editor

Douglas S. Clark

Department of Chemical Engineering, University of California  
Berkeley, California

## Associate Editors

Michael J. Betenbaugh  
Johns Hopkins University  
Baltimore, Maryland  
James D. Bryers  
University of Washington  
Seattle, Washington  
Pauline Doran  
University of New South Wales  
Sydney, Australia  
Jonathan S. Dordick  
Rensselaer Polytechnic Institute  
Troy, New York  
Vassily Hatzimanikatis  
Northwestern University  
Evanston, Illinois  
Charles A. Haynes  
University of British Columbia  
Vancouver, BC, Canada  
Jeffrey A. Hubbell  
ETH—Zürich and University of Zürich  
Zürich, Switzerland

Sang Yup Lee  
Korea Advanced Institute of Science and  
Technology  
Daejeon, Korea  
Lee R. Lynd  
Dartmouth College  
Hanover, New Hampshire  
Tadashi Matsunaga  
Tokyo University of Agriculture and  
Technology  
Tokyo, Japan  
Jens Nielsen  
Technical University of Denmark  
Lyngby, Denmark  
Govind Rao  
Baltimore, Maryland

## Advisory Board

Harvey W. Blanch  
University of California  
Berkeley, California

Ho Nam Chang  
Korea Advanced Institute of Science &  
Technology  
Taejon, Korea  
George Georgiou  
University of Texas  
Austin, Texas  
Isao Karube  
University of Tokyo  
Tokyo, Japan  
Alexander M. Klibanov  
Massachusetts Institute of Technology  
Cambridge, Massachusetts  
Maria-Regina Kula  
Heinrich-Heine Universität Düsseldorf in der  
KFA Jülich, Germany  
Douglas A. Laufkenburger  
Massachusetts Institute of Technology  
Cambridge, Massachusetts  
Bernhard O. Palsson  
University of California at San Diego  
La Jolla, California  
Eleftherios T. Papoutsakis  
Northwestern University  
Evanston, Illinois  
Michael L. Shuler  
Cornell University  
Ithaca, New York  
John Villadsen  
Technical University of Denmark  
Lyngby, Denmark  
Daniel I.C. Wang  
Massachusetts Institute of Technology  
Cambridge, Massachusetts

## Editorial Board

Mohamed Al-Rubeai  
Birmingham, United Kingdom  
Kristi Anseth  
Boulder, CO, USA  
Jun-ichi Anzai  
Sendai, Japan

Mark Arnold  
Iowa City, IA, USA  
William Bentley  
College Park, MD, USA  
Michael Butler  
Winnipeg, AB, Canada  
Manuel J.T. Carrondo  
Oeiras, Portugal  
Jeffrey Chalmers  
Columbus, OH, USA  
Howard Chase  
Cambridge, United Kingdom  
Julian Chaudhuri  
Bath, United Kingdom  
Wilfred Chen  
Riverside, CA, USA  
Bruce Dale  
East Lansing, MI, USA  
Scott Diamond  
Philadelphia, PA, USA  
Erik J. Fernandez  
Charlottesville, VA, USA  
Martin Fussenegger  
Zürich, Switzerland  
Peter Halling  
Glasgow, United Kingdom  
Hiroyuki Honda  
Nagoya, Japan  
Beth Junker  
Rahway, NJ, USA  
Michael Ladisch  
West Lafayette, IN, USA  
Abraham Lenhoff  
Newark, DE, USA  
Cunnar Lidén  
Lund, Sweden  
Robert Lortie  
Montreal, Quebec, Canada  
Brian McNeil  
Glasgow, United Kingdom  
William M. Miller  
Evanston, IL, USA

Alvin Nienow  
Birmingham, United Kingdom  
Henrik Pedersen  
Piscataway, NJ, USA  
James Petersen  
Pullman, WA, USA  
James M. Piret  
Vancouver, BC, Canada  
Octavio T. Ramirez  
Cuernavaca, Mexico  
Joseph O. Rich  
Alt. Prospect, IL, USA  
Anne Skaja Robinson  
Newark, DE, USA  
David Schaffer  
Berkeley, CA, USA  
Masayasu Suzuki  
Toyama, Japan  
Vytas Svedas  
Moscow, Russia  
Masahiro Takagi  
Ishikawa, Japan  
Owen Thomas  
Lyngby, Denmark  
Nigel Titchener-Hooker  
London, United Kingdom  
Luuk van der Wielen  
Delft, The Netherlands  
Mark van Loosdrecht  
Delft, The Netherlands  
William R. Wagner  
Pittsburgh, PA, USA  
Ping Wang  
Akron, OH, USA  
Mark Worden  
East Lansing, MI, USA  
Charles E. Wyman  
Hanover, NH, USA  
Jian-Jiang Zhong  
Shanghai, China  
Andrew Zydney  
State College, PA, USA

Founding Editor: Elmer L. Gaden, Jr., University of Virginia, Charlottesville, Virginia

Biotechnology and Bioengineering (Print ISSN: 0006-3592; Online ISSN: 1097-0290 at Wiley InterScience, [www.interscience.wiley.com](http://www.interscience.wiley.com)) Volumes 93–95 are published 18 times a year, semi-monthly, by Wiley Subscription Services, Inc., a Wiley Company, 111 River Street, Hoboken, NJ 07030.

Copyright © 2006 Wiley Periodicals, Inc., a Wiley Company. All rights reserved. No part of this publication may be reproduced in any form or by any means, except as permitted under section 107 or 108 of the 1976 United States Copyright Act, without written permission of the publisher, authorization through the Copyright Clearance Center, 222 Rosewood Drive, Danvers, MA 01923, 978-750-8400, fax 978-750-4470. Periodicals postage paid at Hoboken, NJ and at additional mailing offices.

The copyright notice appearing at the bottom of the first page of an article in the journal indicates the copyright holder's consent that copies may be made for personal or internal use, or for the personal or internal use of specific clients, on the condition that the copier pay for copying beyond that permitted by Sections 107 and 108 of the United States Copyright Law. The per copy fee for each item is to be paid through the Copyright Clearance Center. This consent does not extend to other kinds of copying, such as copying for general distribution, for advertising or promotional purposes, for creating new collective works, or for resale. Such permission requests and other permission inquiries should be addressed to the Permissions Department, c/o John Wiley & Sons, Inc., 111 River Street, Hoboken, NJ 07030, (201) 748-6011, fax (201) 748-6008, <http://www.wiley.com/go/permissions>.

Subscription price (Volumes 93–95): Print only \$595.00 in U.S., \$6211.00 in Canada and Mexico, \$6337.00 outside North America. Electronic only \$595.00 worldwide. A combination price of \$6594.50 in U.S., \$6810.50 in Canada and Mexico, \$6936.50 outside North America, includes the subscription in both electronic and print formats. Personal rates: \$750.00 in North America, and \$918.00 rest of world. Subscriptions at the personal rate are available to individuals. All subscriptions containing a print element, shipped outside U.S., will be sent by air. Payment must be made in U.S. dollars drawn on U.S. bank. Claims for undelivered copies will be accepted only after the following issue

has been delivered. Please enclose a copy of the mailing label. Missing copies will be supplied when losses have been sustained in transit and where reserve stock permits. Please allow four weeks for processing a change of address. For subscription inquiries, please call (201) 748-6645; E-mail: [SUBINFO@wiley.com](mailto:SUBINFO@wiley.com).

Postmaster: Send address changes to Biotechnology and Bioengineering, Subscription Distribution, c/o John Wiley & Sons, Inc., 111 River Street, Hoboken, NJ 07030.

Advertising Sales: Inquiries concerning advertising should be forwarded to Advertising Sales, c/o John Wiley & Sons, Inc., 111 River Street, Hoboken, NJ 07030; (201) 748-8832.

Reprints: Reprint sales and inquiries should be directed to the Customer Service Department, c/o John Wiley & Sons, Inc., 111 River Street, Hoboken, NJ 07030. Tel: (201) 748-8789.

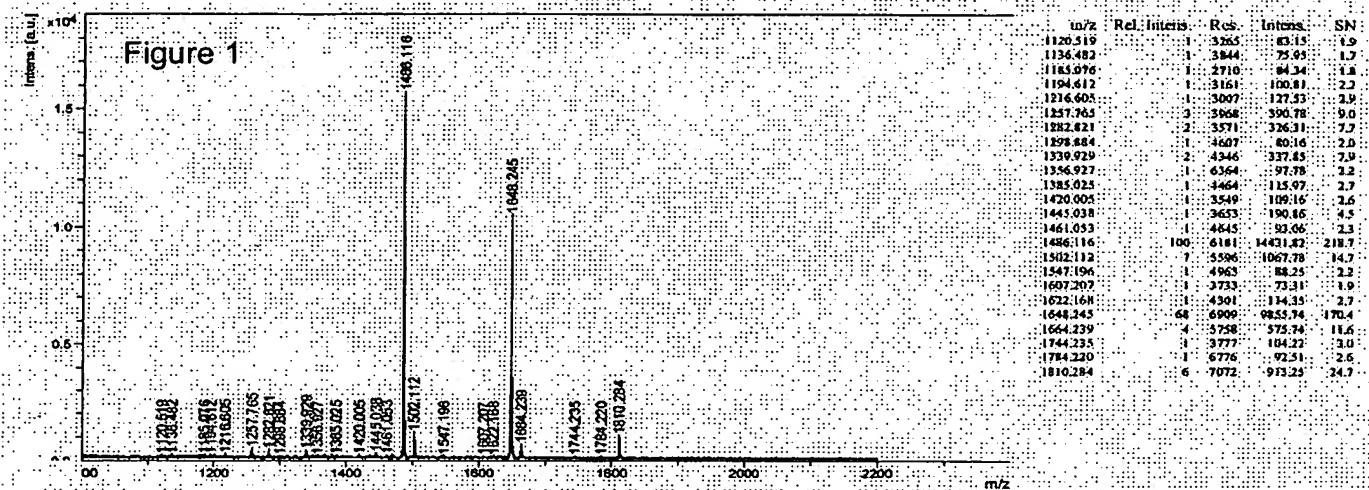
Manuscripts should be submitted to Biotechnology and Bioengineering, Editorial Receiving Office, c/o John Wiley & Sons, Inc., 111 River Street, Hoboken, NJ 07030. Phone: (201) 748-6297; Fax: (201) 748-8881; E-mail: [biotechbioeng@wiley.com](mailto:biotechbioeng@wiley.com)

All Other Correspondence: Address to Biotechnology and Bioengineering, Publisher, c/o John Wiley & Sons, Inc., 111 River Street, Hoboken, NJ 07030.

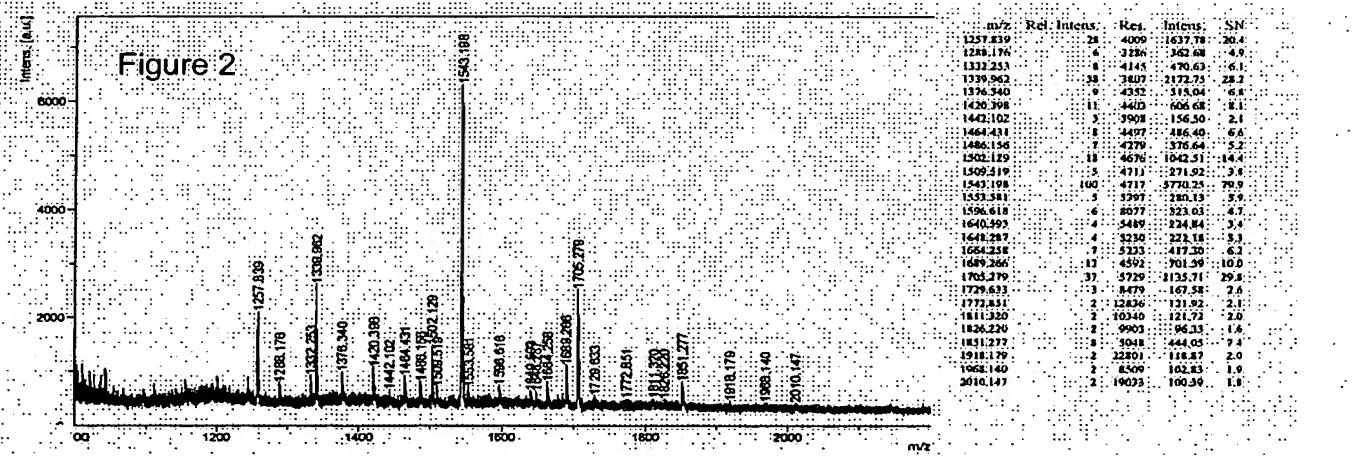
The contents of this journal are indexed or abstracted in Analytical Abstracts, BIOBASE, BIOSIS Previews, Biological Abstracts, Biological and Agricultural Index Plus, Biotechnology Citation Index™, CAB Abstracts, Cambridge Scientific Abstracts, Chemical Abstracts Service/SciFinder, COMPENDEX, Current Awareness in Biological Sciences, Current Contents/Agriculture, Biology, and Environmental Science, Current Contents/Life Sciences, EMBASE/Excerpta Medica, International Abstracts in Operations Research (IAOR), IAOR-Online, ISI Alerting Services (includes Research Alert™, Reference Update™, Science Citation Index™, SciSearch™ (also known as Science Citation Index-Expanded) SCOPUS, and SIIC Databases).

Editorial Production, John Wiley  
Jeffrey Collins

**Antibody glycoengineering by constitutive co-expression of recombinant, wild-type  $\beta$ 1,4-N-acetylglucosaminyltransferase III (GnT-III) and Golgi  $\alpha$ -mannosidase II (ManII) in stable, industrial grade CHO cells producing a recombinant antibody**

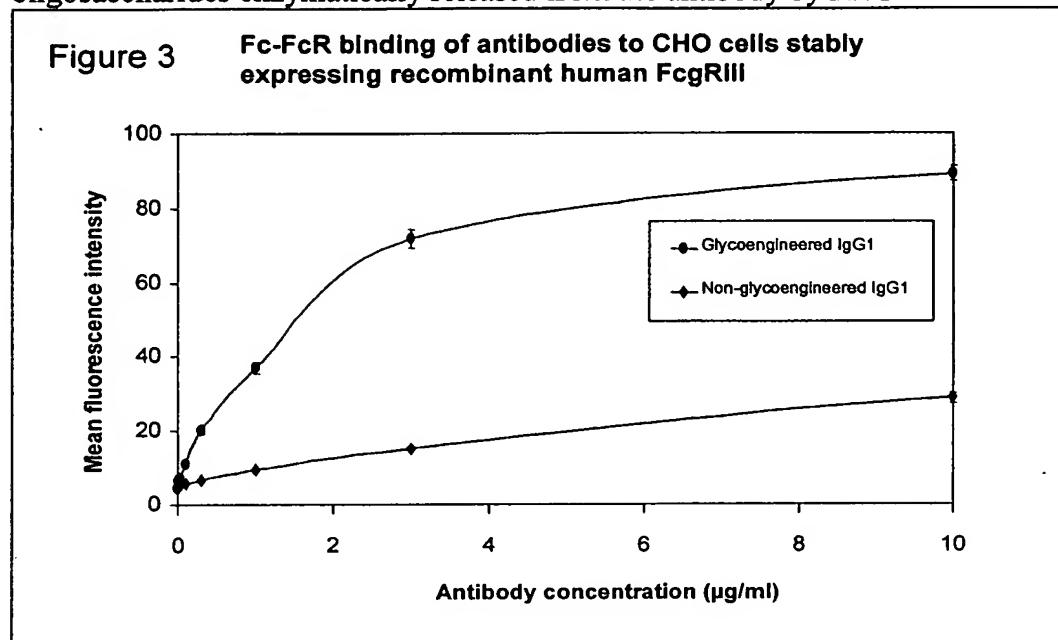


**Figure 1** shows the glycosylation profile for the Fc-region oligosaccharides of a **non-glycoengineered recombinant antibody** produced in CHO cells. The three major peaks ( $m/z$  1486, 1648 and 1810) correspond to fucosylated complex oligosaccharides, and level of non-fucosylated oligosaccharides is below 10%, which is typical for non-glycoengineered antibodies produced by CHO cells. The oligosaccharide profile was determined by MALDI/TOF-MS in positive ion mode for oligosaccharides enzymatically released from the antibody by PNGaseF treatment.

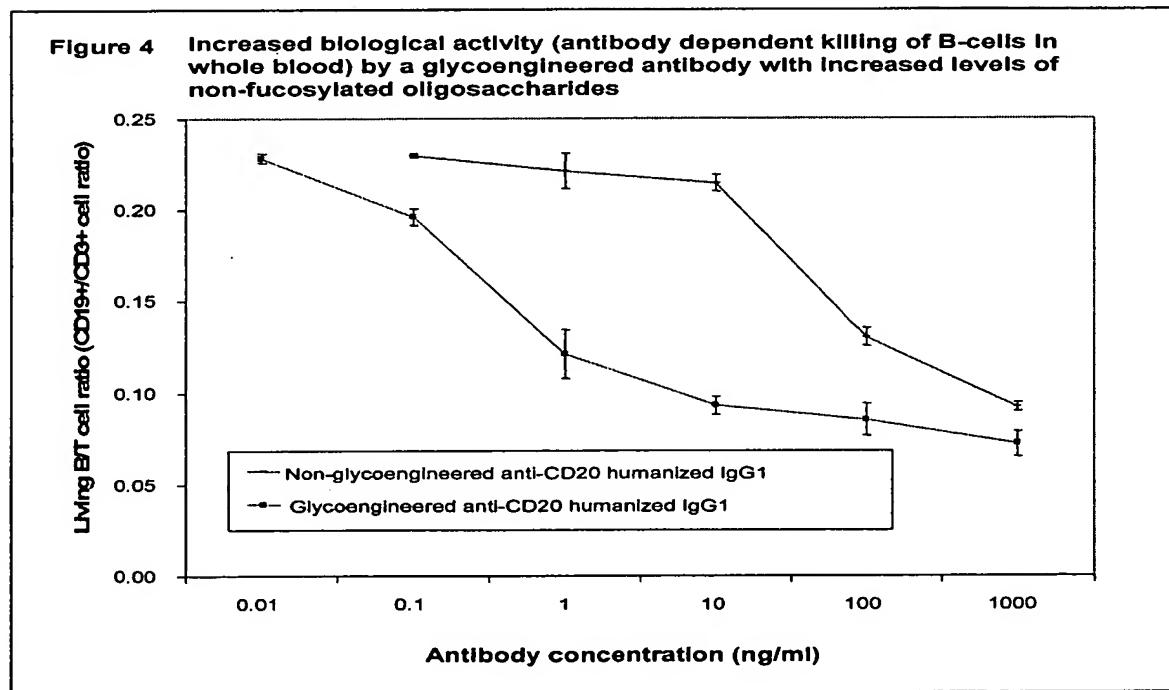


**Figure 2** shows the glycosylation profile for the Fc-region oligosaccharides of a **glycoengineered recombinant antibody** produced in CHO cells. Glycoengineering was achieved by constitutive co-expression of recombinant, wild-type  $\beta$ 1,4-N-acetylglucosaminyltransferase III (GnT-III) and Golgi  $\alpha$ -mannosidase II (ManII) in stable, industrial grade CHO cells producing a recombinant antibody. Industrial grade means that the cells grow (with a doubling time lower than 40 hours) in suspension in chemically-defined protein-free medium and produce over 0.5 g/l of antibody under batch process conditions. Glycoengineering leads to increased levels of non-fucosylated oligosaccharides relative to levels produced in non-glycoengineered CHO cells (peaks at  $m/z$  1339, 1543 and 1705 are

non-fucosylated complex oligosaccharides and represent over 70% of the oligosaccharides). The oligosaccharide profile was determined by MALDI/TOF-MS in positive ion mode for oligosaccharides enzymatically released from the antibody by PNGaseF treatment.



**Figure 3** shows increased binding to FcgRIII for a glycoengineered antibody, having increased levels of non-fucosylated Fc-oligosaccharides (glycoengineering as described above for Figure 2), relative to a non-glycoengineered antibody with identical Fc polypeptide region. Binding assay was performed as described in



**Figure 4** shows increased antibody-dependent killing of target cells for a glycoengineered antibody with increased levels of non-fucosylated Fc-oligosaccharides (glycoengineered as

described above for Figure 2) relative to a non-glycoengineered antibody with identical Fc polypeptide region. B-cell antibody-dependent-depletion assay performed by incubating antibodies with whole blood overnight and measuring the levels of remaining, living B-cells by flow cytometry (T-cells are used as an internal standard that is not depleted by the anti-CD20 antibodies). Assay details are as described in

- -

More specifically, the present invention is directed to a method for producing an altered glycoforms of proteins having improved therapeutic values, e.g., an antibody which has an enhanced antibody dependent cellular cytotoxicity (ADCC), in a host cell. The invention provides host cells which harbor a nucleic acid encoding the protein of interest, e.g., an antibody, and at least one nucleic acid encoding a glycosyl transferase. Further, the present invention provides methods and protocols of culturing such host cells under conditions which permit the expression of said protein of interest, e.g., the antibody having enhanced antibody dependent cellular cytotoxicity. Further, methods for isolating the so generated protein having an altered glycosylation pattern, e.g., the antibody with enhanced antibody dependent cellular cytotoxicity, are described.

Furthermore, the present invention provides alternative glycoforms of proteins having improved therapeutic properties. The proteins of the invention include antibodies with an enhanced antibody-dependent cellular cytotoxicity (ADCC), which have been generated using the disclosed methods and host cells.

#### IV. BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 depicts structures of common types of N-linked oligosaccharides and nomenclature. M stands for mannose; Gn, N-acetylglucosamine (GlcNAc); G, galactose; Gn<sup>b</sup>, bisecting GlcNAc; R, Asn-Gn- $\beta$ 1,4-Gn or Asn-Gn- $\beta$ 1,4-( $\alpha$ 1,6-fucose)-Gn. R-M is called the oligosaccharide "core". The square brackets indicated that at least one Gn is linked to a G. the oligosaccharide nomenclature used in this work consists of enumerating the M and Gn residues attached to the R group, indicating the presence of a bisecting GlcNAc by including a Gn<sup>b</sup>, and indicating if the oligosaccharide is galactosylated by including a G. The two types of tri-antennary oligosaccharides are differentiated by addint an apostrophe to the Gn<sub>3</sub> term.

FIGURE 2 depicts central reaction network on the N-linked glycosylation pathway. This set of Golgi-localized reactions determines the major types of structures into which N-linked oligosaccharides are normally classified. The enzyme catalyzing each reaction is shown and all reactions have been numbered. The reaction numbers are used to denote the kinetic parameters associated with each reaction (see, TABLE IV for example).

B. A Mathematical Model Of N-Linked Glycoform Biosynthesis

1. Overview

Metabolic engineering of N-linked oligosaccharide biosynthesis to produce novel glycoforms or glycoform distributions of a recombinant glycoprotein can potentially lead to an improved therapeutic performance of the glycoprotein product. Effect engineering of this pathway to maximize the fractions of beneficial glycoforms within the glycoform population of a target glycoprotein can be aided by a mathematical model of the N-linked glycosylation process. A mathematical model is presented here, whose main function is to calculate the expected qualitative trends in the N-linked oligosaccharide distribution resulting from changes in the levels of one or more enzymes involved in the network of enzyme-catalyzed reactions which accomplish N-linked oligosaccharide biosynthesis. It consists of mass balances for 33 different oligosaccharide species N-linked to a specific protein that is being transported through the different compartments of the Golgi complex. Values of the model parameters describing Chinese hamster ovary (CHO) cells were estimated from literature information. A basal set of kinetic parameters for the enzyme-catalyzed reactions acting on free oligosaccharide substrates was also obtained from the literature. The solution of the system for this basal set of parameters gave a glycoform distribution consisting mainly of complex-galactosylated oligosaccharides, distributed in structures with different numbers of antennae in a fashion similar to that observed for various recombinant proteins produced in CHO cells. Other simulations indicate that changes in the oligosaccharide distribution could easily result from alteration in glycoprotein productivity within the range currently attainable in industry. The overexpress of N-acetylglucosaminyltransferase III (GnT III) in CHO cells was simulated under different conditions to test the main function of the model. These simulations allow a comparison of different strategies, such as simultaneous overexpression of several enzymes or spatial relocation of enzymes, when trying to optimize a particular glycoform distribution.

The mathematical model disclosed herein consists of mass balances for 33 different oligosaccharide species N-linked to a specified protein that is being transported through the different compartments of the Golgi complex. These equations relate the oligosaccharide mole fractions to the amounts of the different enzymes, the kinetic

constants of the reactions, the distribution of enzymes in the different compartments, the half-life of the protein in the Golgi, the volume of the compartments, and the specific glycoprotein productivity. Values for the parameters in the model and their normal ranges can either be found in the literature or estimated from literature information.

5 Some of the parameters are specific for each cell line. Those describing Chinese hamster ovary (CHO) cells were used here, since CHO cells are currently the most common host for the industrial production of therapeutic glycoproteins. Numerical simulations of the model with these values of the parameters gave glycoform distributions similar to those observed for some proteins produced in CHO cells.

10 One characteristic of the glycosylation pathway makes its modelling different from that of other biochemical pathways. Oligosaccharides have some degree of conformational flexibility and, through interactions with the polypeptide chain, certain conformations can be preferentially stabilized. Wyss and Gerhard, 1996, *Current Opinion Biotechnol.* 7:409-416. In addition, the polypeptide backbone around the glycosylation site may limit the access of the catalytic sites of the enzymes to the oligosaccharide. Shao and Wold, 1995, *Eur. J. Biochem.* 228:79-85. As a result, a particular glycosylation site can have its own set of values for the kinetic constants of the enzyme-catalyzed reactions. These values can be different from those of other glycosylation sites in the same or other proteins. The occurrence of this phenomenon can be inferred from the numerous examples where very different oligosaccharide distributions have been observed for different glycosylation sites of the same protein, even though all other system variables were identical for all sites during biosynthesis. Nevertheless, the range of values of the kinetic constants for oligosaccharides on some glycoproteins lies close to the corresponding range for soluble oligosaccharides (Do *et al.*, 1994, *J. Biol. Chem.* 269:23456-23464; Rao and Mendicino, 1978, *Biochemistry* 17:5635-5638; Gross *et al.*, 1990, *Anal. Biochem.* 186:127-134. Motivated by this observation, the constants for the latter were used as an initial approach to test the model and to study some aspects of its general behavior.

5            2. Physical Model

The N-linked glycosylation pathway consists of enzyme-catalyzed reactions which first attach a common oligosaccharide precursor to appropriate glycosylation sites in a polypeptide and then modify the linked oligosaccharides to produce a heterogenous set of glycoforms. Kornfeld and Kornfeld, 1985, *Annu. Rev. Biochem.* **54**:631-664. Potential glycosylation sites are asparagine residues in the sequence Asn-X-Ser/Thr. The reactions take place in the endoplasmic reticulum (ER) and in the Golgi complex (Golgi) as proteins are transported through these cellular compartments *en route* to their final destinations. These destinations may be, for example, the ER or Golgi themselves, the plasma membrane, or the extracellular space.

10            The initial covalent attachment of the oligosaccharide precursor to the protein takes place during translocation of the latter into the lumen of the ER. Not all the translocated molecules acquire oligosaccharides in their potential glycosylation sites, and the fraction that does may vary between sites. The type of glycoform heterogeneity which thus results is called glycoform macro-heterogeneity. Shelikoff *et al.*, 1996, *Biotechnol. Bioeng.* **50**:73-90. Once in the ER, the N-linked oligosaccharides are trimmed down by glycosidases which can sequentially remove three molecules of glucose and, sometimes, one of mannose. The glycoproteins are then transported to the Golgi where a different set of glycosidases and glycosyltransferases act on the N-linked oligosaccharides and lead to a diversity of structures. Such type of heterogeneity in the identity of the attached oligosaccharides is referred to as glycoform micro-heterogeneity.

15            A mathematical model of glycoform macro-heterogeneity has been published recently. Shelikoff *et al.*, 1996, *supra*. It incorporates different factors that determine the extent of the first transfer reaction of the pathway. In contrast, the model presented below is concerned with glycoform micro-heterogeneity. More specifically, it deals with a set of eight Golgi-localized enzymes which together determine the distribution of oligosaccharides into the following major structural classes: high mannose, hybrid, bi-, tri-, tri'- and tetra-antennary complex, bisected hybrid, and bisected bi-, tri-, tri'-, and tetra-antennary complex oligosaccharides (FIGURES 1 and 2). Thirty-three different oligosaccharide species are involved in 33 reactions catalyzed by these enzymes; including 5 high-mannose, 3 hybrid, 3 hybrid-galactosylated, 4 complex (bi-, tri-, tri',

and tetra-antennary complex), 4 complex-galactosylated, and the 14 bisected counterparts of the hybrid and complex oligosaccharides. The products of this set of reactions can be processed further in the Golgi through more transferase-catalyzed reactions that increase glycoform micro-heterogeneity.

5       The major elements of the physical model are: (a) the different Golgi compartments where the reactions take place and the transport of proteins between them, (b) the central network of enzyme-catalyzed reactions, and (c) the spatial distribution of these enzymes in the different Golgi compartments.

10      The Golgi complex consists of a series of distinct, membrane-bounded compartments. Proteins destined to the extracellular space, plasma membrane, lysosomes, endosomes, or secretory storage vesicles are transported from the ER to the first Golgi compartment, the cis-Golgi network (CGN). From there, they travel in sequential order through the remaining compartments of the series; the cis-medial-, and trans-Golgi cisternae, which together comprise the Golgi stack; and then to the trans-Golgi network (TGN), the final sorting place. Rothman and Orci, 1992, *Nature* 355:409-415. There is some controversy about the number of cisternae in the Golgi stack, but in the present model only three are considered.

20      Proteins are transported between compartments by vesicles which bud off from the membrane of one compartment and fuse to the next in the series. Rothman and Wieland, 1996, *Science* 272:227-234. Secreted and plasma membrane proteins appear to go through the Golgi by a "bulk flow" mechanism. These proteins enter vesicles by default, *i.e.*, in the absence of specific transport or retention signals, and therefore at their bulk concentration in the donor compartment. Proteins which reside in the ER or Golgi require retention signals that allow them to be concentrated in the appropriate compartments. Such residency is not permanent and their relative concentration in a particular region is also aided by retrieval-vesicles that recognize transport signals in escaped proteins and return them to previous compartments.

25      For the physical model, four of the five Golgi compartments mentioned above are considered as a system of four reactors in series. The modelled compartments are the cis-, medial-, and trans-Golgi cisternae, and the TGN. This selection is based on immuno-electron microscopy studies that localize the enzymes included in the present

model to these compartments. Nilsson *et al.*, 1993, *J. Cell. Biol.* 120:5-13; Rabouille *et al.*, 1995, *J. Cell Science* 108:1617-1627. The chemical reactions catalyzed by these enzymes are described next.

5           Central Reaction-Network. The N-linked glycosylation pathway of mammalian cells has been deduced by a combination of *in vitro* and *in vivo* biosynthetic studies. Kornfeld and Kornfeld, 1985, *supra*; Schachter, 1986, *Biochem. Cell Biol.* 64:163-181. Although many enzymes participate in the pathway, a subset of them determines the distribution of oligosaccharides into 33 different species which together define the high mannose, hybrid, hybrid-bisected, complex and complex-bisected types. The network of 10 reactions catalyzed by this subset is called the "central reaction network" (CRN). The CRN considered in the present model is depicted in FIGURE 2.

The first enzyme of the CRN is Golgi  $\alpha$ 1,2-mannosidase I (Man I), which can cleave  $\alpha$ 1,2-linked mannose residues from  $M_9$ - $M_6$  to finally produce  $M_5$  (*see*, nomenclature in FIGURE 1), corresponding to reactions 1 to 4 in FIGURE 2. All eukaryotic cells have an  $\alpha$ 1,2-mannosidase in the ER that can also catalyze reaction 1. Therefore, the initial substrate for the Golgi CRN is a mixture of  $M_9$  and  $M_8$  oligosaccharides. Compounds  $M_9$  to  $M_5$  constitute the high-mannose class of N-linked oligosaccharides. The synthesis of hybrid and complex oligosaccharides then follows as described above.

An N-acetylglucosamine (GlcNAc) can be transferred to the  $\alpha$ 1,3-mannose branch of  $M_5$  by  $\beta$ 1,2-N-acetylglucosaminyltransferase I (GnT I) to yield  $M_5$ Gn, the first hybrid oligosaccharide.  $M_5$ Gn is a substrate for  $\alpha$ -mannosidase II (Man II), which catalyzes the removal of two mannose residues resulting in hybrids  $M_4$ Gn (Reaction 6) and  $M_3$ Gn (reaction 7). The free  $\alpha$ 1,6-mannose branch of  $M_3$ Gn is then available for extension by GnT II to produce  $M_3$ Gn<sub>2</sub>, a complex bi-antennary oligosaccharide.  $M_3$ Gn<sub>2</sub> may be branched further by GnT IV or GnT V. GnT IV adds a GlcNAc in a  $\beta$ 1,4-linkage to the  $\alpha$ 1,6-mannose branch, leading to the tri-antennary complex oligosaccharide  $M_3$ Gn<sub>3</sub>. GnT V catalyzes a GlcNAc transfer in a  $\beta$ 1,6-linkage to the  $\alpha$ 1,3-mannose branch and produces the tri'-antennary complex oligosaccharide  $M_3$ Gn<sub>3'</sub>. The tetra-antennary complex compound  $M_3$ Gn<sub>4</sub> can be synthesized both by GnT IV from  $M_3$ Gn<sub>3'</sub> (reaction 11) and by GnT V from  $M_3$ Gn<sub>3</sub> (reaction 12).

5

All hybrid and complex oligosaccharides contain non-reducing-end GlcNAcs which may be extended by  $\beta$ 1,4-galactosyltransferase (GalT, reaction 13 to 19). Once a galactose residue is transferred, the modified oligosaccharide is no longer a biosynthetic substrate for any of the remaining GnTs or for Man II. Schachter, 1986, *supra*. All of the branches in any complex oligosaccharide serve as substrates for GalT, but do so with different affinities. Pacquet *et al.*, 1984, *J. Biol. Chem.* 259:4716-4721. In the present model, these reactions are lumped together in single steps which remove the compound from the flux through reactions 1 to 12.

10

on  
0  
0  
0  
0  
E  
0  
0  
P  
= 0  
0  
F  
0  
0  
20  
0  
0  
0

The reactions mentioned to this point take place in common industrial cell lines, such as CHO cells and baby hamster kidney (BHK) cells, used for the production of recombinant glycoproteins. Jenkins *et al.*, 1996, *Nature Biotechnol.* 14:975-981. An additional set of reactions (20 to 33) is also important for determining the major classes of N-linked oligosaccharides in cell lines expressing GnT III. Examples of these cell lines are a glycosylation mutant of CHO cells named Lec 10 (Stanley and Campbell, 1984, *J. Biol. Chem.* 261:13370-13378) and rat myeloma (Y0) cells (Lifely *et al.*, 1995, *Glycobiology* 318:813-822. As indicated in reactions 20 to 26, GnT III can modify any non-galactosylated hybrid or complex oligosaccharide by transferring a GlcNAc residue in a  $\beta$ -1,4-linkage to the core mannose. The transferred residue is called a bisecting GlcNAc (Gn<sup>b</sup>), and the products of these reactions are referred to as bisected oligosaccharides. GalT cannot extend the Gn<sup>b</sup> residue, but it may modify all the other non-reducing-end GlcNAcs of any bisected oligosaccharide (reactions 27 to 33).

25

The final products of the CRN are usually modified further in the Golgi by the addition of sialic acids, poly-N-acetyllactosamine, fucose, N-acetylgalactosamine, sulphate, and  $\alpha$ 1,3-linked galactose. Wild type CHO cells only add sialic acids (in  $\alpha$ 2,3-linkages to galactose), fucose ( $\alpha$ 1,6-linked to the oligosaccharide core, *see*, R in FIGURE 1), and poly-N-acetyllactosamine (to various antennae but preferentially to that synthesized by GnT V). The addition of fucose to the core of oligosaccharides can take place at any point after reaction 5 of the CRN, but it is also blocked by the modifications that GalT or GnT III introduce. Core-fucosylated oligosaccharides can go through the rest of the CRN in the same way as their non-fucosylated counterparts,

Sugars  
Glc  
Gal  
GlcNAc  
GalNAc

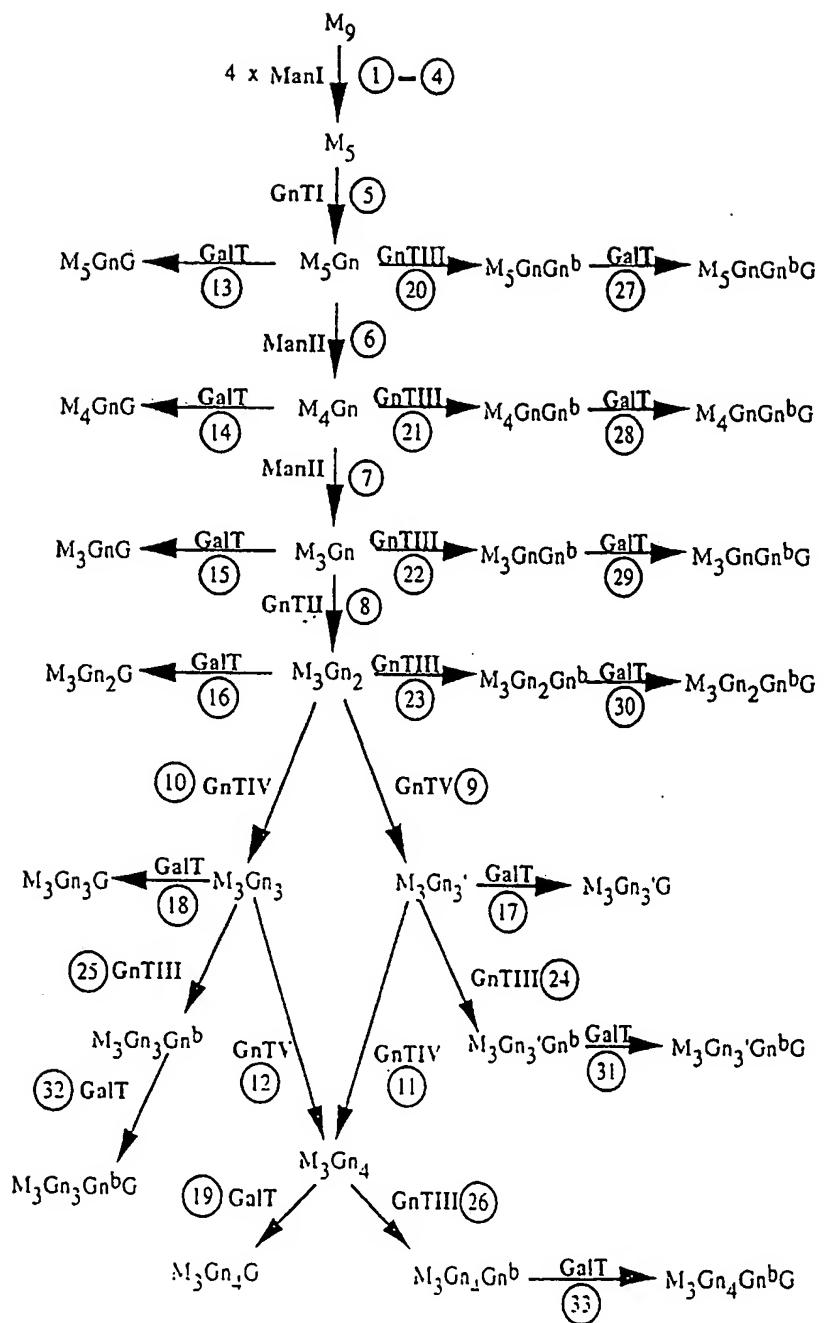


FIGURE 2

ANNALS OF THE NEW YORK ACADEMY OF SCIENCES

*Volume 745*

## BIOCHEMICAL ENGINEERING VIII

*Edited by Robert M. Kelly, K. Dane Wittrup, and Subhash Karkare*



*The New York Academy of Sciences  
New York, New York  
1994*

*Copyright © 1994 by the New York Academy of Sciences. All rights reserved. Under the provisions of the United States Copyright Act of 1976, individual readers of the Annals are permitted to make fair use of the material in them for teaching and research. Permission is granted to quote from the Annals provided that the customary acknowledgment is made of the source. Material in the Annals may be republished only by permission of the Academy. Address inquiries to the Executive Editor at the New York Academy of Sciences.*

*Copying fees: For each copy of an article made beyond the free copying permitted under Section 107 or 108 of the 1976 Copyright Act, a fee should be paid through the Copyright Clearance Center, Inc., 222 Rosewood Drive, Danvers, MA 01923. For articles of more than 3 pages, the copying fee is \$1.75.*

*© The paper used in this publication meets the minimum requirements of American National Standard for Information Sciences—Permanence of Paper for Printed Library Materials, ANSI Z39.48-1984.*

**Library of Congress Cataloging-in-Publication Data**

Biochemical engineering VIII / edited by Robert M. Kelly, K. Dane Wittrup, and Subhash Karkare.  
p. cm. — (Annals of the New York Academy of Sciences, v. 745)  
Includes bibliographical references and index.  
ISBN 0-89766-913-4 (cloth) — ISBN 0-89766-914-2 (paper)  
1. Biochemical engineering—Congresses. I. Kelly, Robert M., 1953- II. Wittrup, K. Dane. III. Karkare, Subhash B. IV. Biochemical Engineering Conference (8th : 1993 : Princeton, N.J.) V. Series.  
Q11.N5 vol. 745  
[TP248.3]  
500 s—dc20  
[660.'63]

94-41383  
CIP

&JOS

*Printed in the United States of America*  
ISBN 0-89766-913-4 (cloth)  
ISBN 0-89766-914-2 (paper)  
ISSN 0077-8923

# **Alteration of Oligosaccharide Biosynthesis by Genetic Manipulation of Glycosyltransferases<sup>a</sup>**

ADEL YOUAKIM AND BARRY D. SHUR

*Department of Biochemistry and Molecular Biology*

*Box 117*

*University of Texas M.D. Anderson Cancer Center*

*1515 Holcombe Boulevard*

*Houston, Texas 77030*

## **INTRODUCTION**

Carbohydrate chains can directly mediate or modulate the function of glycoproteins in diverse biological processes.<sup>1</sup> The ability to manipulate the oligosaccharide structures of glycoproteins in order to alter their biological properties would be of obvious value. This is particularly relevant with regard to biologically important molecules such as growth factors, hormones, and other therapeutic agents that are being produced in cultured cells. Altering the sugar chains of these glycoproteins may improve their therapeutic value by increasing their efficacy, altering their circulatory half-lives, and/or increasing their target specificity. In addition, altered glycosylation of cell-surface components may provide insight to the precise roles that cell-surface glycoconjugates play in processes such as migration, adhesion, development, and malignancy.

Several methods have evolved to alter glycosylation in cells. These have included the use of reagents that inhibit glycosylation as well as inhibitors of glycosylation processing.<sup>2</sup> These inhibitors have been widely used to study the sugar chains of glycoconjugates, but many of these reagents are toxic to cells, and in some instances, their effects are only partial. Another approach has been to use mutagenized cells that are resistant to the toxic effects of specific lectins due to deficiencies in corresponding glycosylation reactions.<sup>3</sup> One limitation of this approach is that mutants are not generated at each step of the biosynthetic pathway. In addition, both of these approaches are of limited use for obtaining large, complex oligosaccharides, inasmuch as they result in the formation of incomplete or truncated carbohydrate structures.

One method that overcomes these limitations and that allows one to selectively manipulate oligosaccharide structure is to express cloned genes for glycosyltransferases into mammalian cells. As more genes for these enzymes are cloned, the possibilities for altering the biosynthetic pathways of oligosaccharide in cells could be substantial. It is this new approach that will be the focus of this review.

<sup>a</sup>This work was supported by NIH Grant HD 22590 to Barry D. Shur.

## GLYCOSYLTRANSFERASES

Glycosyltransferases are enzymes localized to the endoplasmic reticulum and Golgi complex of all mammalian cells. These enzymes catalyze the transfer of sugars donated from either lipid or nucleotide intermediates to mono- and oligosaccharide acceptors. To date, more than a dozen different glycosyltransferases have been cloned and characterized.<sup>4</sup> Although some of these enzymes share common substrates, they show very little sequence homology. The topology and domain structures of all the cloned glycosyltransferases, however, are remarkably similar in that all are type II membrane glycoproteins containing short cytoplasmic domains and large luminal catalytic domains. The availability of cDNAs for many of these enzymes makes it possible to reintroduce them into host cells and to subsequently affect glycosylation.

Alteration of glycosylation through genetic manipulation of glycosyltransferases can be classified into two categories: the expression of glycosyltransferases that are not normally active in the host cells (exogenous glycosyltransferases), and over-expression of glycosyltransferases in host cells containing endogenous activities of these enzymes (endogenous glycosyltransferases). The first approach has been the most extensively studied because screening for alterations in glycosylation is relatively simple, requiring the detection of novel oligosaccharides on the cell surface using antibody and/or lectin probes. The second approach, on the other hand, requires more rigorous quantitative analysis because increasing the levels of glycosyltransferases would most likely not generate different types, but rather, different amounts of oligosaccharide structures.

## GENETIC MANIPULATION OF GLYCOSYLTRANSFERASE EXPRESSION

### *Exogenous Glycosyltransferases*

With few exceptions, the introduction of exogenous glycosyltransferases has used enzymes that act on terminal glycosylation, such as fucosylation and sialylation. The following section describes some studies that have used this approach to modify glycosylation.

A.  $\alpha$ 1,3 Fucosyltransferase ( $\alpha$ 1,3 FT) catalyzes the transfer of fucose (Fuc) from GDP-fucose to *N*-acetylglucosamine (GlcNAc) of an *N*-acetyllactosamine unit. Several reports have demonstrated that introducing the cDNA for this glycosyltransferase into Chinese hamster ovary (CHO) cells lacking this activity results in the biosynthesis of fucosylated *N*-acetyllactosamine, also known as the Lewis x ( $Le^x$ ) antigen.<sup>5-7</sup> This oligosaccharide is of biological interest because it is the ligand for the ELAM receptor on endothelial cells and is required for lymphocyte homing. The ability to synthesize large quantities of oligosaccharides and glycoproteins containing  $Le^x$  is of considerable interest, because these oligosaccharides may have potential therapeutic applications in the treatment of inflammation<sup>8</sup> and, perhaps, malignant invasion.<sup>9</sup>

B.  $\alpha$ 1,2 Fucosyltransferase ( $\alpha$ 1,2 FT) catalyzes the transfer of Fuc from GDP-

fucose to galactose (Gal) residues, resulting in the formation of the H blood group antigen. Similar to the studies with  $\alpha$ 1,3 FT, the introduction of the cloned gene for  $\alpha$ 1,2 FT into COS-1 cells, which lack this enzymic activity, results in the formation of H blood group antigen.<sup>10</sup>

C.  $\alpha$ 1,3 Galactosyltransferase ( $\alpha$ 1,3 GT) catalyzes the transfer of Gal from UDP-Gal to terminal Gal residues of oligosaccharide chains. Transfection of  $\alpha$ 1,3 GT into CHO cells results in the expression of  $\alpha$ 1,3 Gal-containing oligosaccharides.<sup>11</sup> Furthermore, the expression of this enzyme results in a concomitant decrease in terminal sialylation of sugar chains in these cells. Thus, this study also shows that competition between different glycosyltransferases can affect glycosylation *in vivo*.

D.  $\alpha$ 2,6 Sialyltransferase ( $\alpha$ 2,6 ST) catalyzes the transfer of sialic acid (SA) from CMP-SA to terminal Gal residues. Transfection of the cDNA encoding this protein into CHO cells results in the expression of appropriately sialylated sugar chains.<sup>12</sup> As was the case for example C above, there is competition for substrates between the transfected sialyltransferase and a similar endogenous enzyme that adds SA in an  $\alpha$ 2,3 linkage to Gal.

E.  $\beta$ 1,3 Galactosyltransferase ( $\beta$ 1,3 GT) catalyzes the transfer of Gal to terminal GlcNAc residues in the biosynthesis of type 1 (Gal  $\beta$ 1,3 GlcNAc) *N*-acetyllactosamine chains. This class of oligosaccharides is of particular biological and structural importance because it serves as the core structure for various blood group activities. Transfection of the cDNA encoding this enzyme into human colonic cells results in the biosynthesis of type 1 *N*-acetyllactosamine-containing oligosaccharides.<sup>13</sup> In addition, many of these chains were further modified with SA and Fuc residues. In contrast to the studies described above, this study is unique in showing that the expression of glycosyltransferase acting early in oligosaccharide biosynthesis (*i.e.*, during elongation) can also alter glycosylation.

#### *Endogenous Glycosyltransferase*

To date, the only example of over-expression of an endogenously expressed glycosyltransferase is that of  $\beta$ 1,4 galactosyltransferase ( $\beta$ 1,4 GT).  $\beta$ 1,4 GT catalyzes the transfer of Gal from UDP-Gal to terminal GlcNAc residues in the biosynthesis of type 2 (Gal  $\beta$ 1,4 GlcNAc) *N*-acetyllactosamine cores of all *N*-linked complex oligosaccharides.  $\beta$ 1,4 GT is unique among the cloned glycosyltransferases in that one gene encodes two similar forms of the enzyme that differ by an additional 13 amino acids at the cytoplasmic N-terminus of the long form that is not present in the short form of the enzyme.<sup>14,15</sup> Both the long and the short form are localized primarily in the Golgi complex. However, the long form of GT is also targeted to the plasma membrane,<sup>15</sup> where it associates with the cytoskeleton and functions as a cell-adhesion molecule.<sup>16</sup>

Because of the central role this enzyme plays in the biosynthesis of *N*-acetyllactosamine-containing oligosaccharides, the effects of overexpressing this enzyme were explored in detail. Transfection of the cDNAs encoding the two forms of  $\beta$ 1,4 GT into F9 embryonal carcinoma cells expressing endogenous enzyme results in a threefold increase in total  $\beta$ 1,4 GT activity compared to control cells.<sup>17</sup> Analysis of [<sup>3</sup>H]Gal-labeled glycoproteins and glycopeptides by a variety of methods revealed

no significant quantitative or qualitative differences in glycosylation. Similar analysis of a specific glycoprotein, lysosomal-associated membrane glycoprotein (LAMP)-1 indicated no differences in glycosylation between the transfected and control cell lines. The inability of transfected  $\beta$ 1,4 GT to affect glycosylation was not due to an absence of GT substrates, inasmuch as an excess of substrate was detected in lysed cells using endogenous and exogenous GT. The transfected GT were properly targeted to the Golgi complex, and, more importantly, were elevated in this compartment in the transfected cells. Thus, in these cells,  $\beta$ 1,4 GT is not rate limiting for oligosaccharide biosynthesis.

### SUMMARY

The alteration of oligosaccharide structures through genetic manipulation of glycosyltransferase activities is now a reality. It is apparent that this technique has greater consequences on oligosaccharide structure when an exogenous enzyme is introduced into cells, and in particular when this enzyme is responsible for a terminal glycosylation step. By contrast, only one study has examined the effects of over-expressing an endogenous glycosyltransferase, in which there was no detectable effect on glycosylation. However, there are still other key regulatory biosynthetic enzymes, such as GlcNAc transferase V and  $\beta$ 1,3 GlcNAc transferase, whose over-expression may alter glycosylation. Both of these enzymes are required for the biosynthesis of polylactosaminoglycans (polymers of *N*-acetyllactosamine disaccharides), and their elevation in tumor cells correlates with increased expression of polylactosaminoglycans.<sup>18,19</sup> Recently, the gene encoding GlcNAc transferase V has been isolated,<sup>20</sup> but its transfection into cells and characterization of the resulting oligosaccharides awaits further study.

Alternate strategies for modifying oligosaccharide structures could involve the introduction of more than one glycosyltransferase into cells to ensure the availability of biosynthetic intermediates. Alternatively, the disruption of specific glycosyltransferase genes by homologous recombination could be used to eliminate competing glycosyltransferases that act on a common substrate.

Although oligosaccharide biosynthesis is directly dependent upon the presence or absence of specific glycosyltransferases, other factors also contribute to glycosylation. For example, the transport rate of a glycoprotein through the endoplasmic reticulum and Golgi complex, the levels of processing glycosidases, the availability of substrates, the host cell, and ultimately, the peptide backbone of the particular glycoprotein of interest are important contributors to the final outcome of oligosaccharide structure. Despite these complications, further study into the manipulation of glycosyltransferase genes may ultimately allow the controlled and predictable biosynthesis of glycoprotein sugar chains.

### REFERENCES

1. VARKI, A. 1993. *Glycobiology* 3: 97–130.
2. ELBEIN, A. 1991. *FASEB J.* 5: 3055–3063.
3. STANLEY, P. 1992. *Glycobiology* 2: 99–107.

4. JOZIASSE, D. H. 1992. *Glycobiology* 2: 271-277.
5. LOWE, J. B., L. M. STOOLMAN, R. P. NAIR, R. D. LARSEN, T. L. BERHEND & R. M. MARKS. 1990. *Cell* 63: 475-484.
6. GOELZ, S. E., C. HESION, D. GOFF, B. GRIFFITHS, R. TIZARD, B. NEWMAN, G. CHI-ROSSO & R. LOBB. 1990. *Cell* 63: 1349-1356.
7. POTVIN, B., R. KUMAR, D. R. HOWARD & P. STANLEY. 1990. *J. Biol. Chem.* 265: 1615-1622.
8. MULLIGAN, M. S., J. C. PAULSON, S. DE-FREES, Z. L. ZHENG & J. B. LOWE. 1993. *Nature* 364: 149-151.
9. SAWADA, R., J. B. LOWE & M. FUKUDA. 1993. *J. Biol. Chem.* 268: 12675-12681.
10. LARSEN, R. D., L. K. ERNST, R. P. NAIR & J. B. LOWE. 1990. *Proc. Natl. Acad. Sci. USA* 87: 6674-6678.
11. SMITH, D. F., R. D. LARSEN, S. MATTOX, J. B. LOWE & R. D. CUMMINGS. 1990. *J. Biol. Chem.* 265: 6225-6234.
12. LEE, E. U., J. ROTH & J. C. PAULSEN. 1989. *J. Biol. Chem.* 264: 13848-13855.
13. SHERWOOD, A. L., T. G. GREENE & E. H. HOLMES. 1992. *J. Cell. Biochem.* 50: 165-177.
14. LOPEZ, L. C., A. YOUAKIM, S. C. EVANS & B. D. SHUR. 1991. *J. Biol. Chem.* 266: 15984-15991.
15. SHAPER, N. L., G. F. HOLLIS, J. G. DOUGLAS, I. R. KIRSCH & J. H. SHAPER. 1988. *J. Biol. Chem.* 263: 10420-10428.
16. EVANS, S. C., L. C. LOPEZ & B. D. SHUR. 1993. *J. Cell Biol.* 120: 1045-1057.
17. YOUAKIM, A. & B. D. SHUR. 1993. *Glycobiology* 3: 155-163.
18. HOLMES, E. H., S. HAKOMORI & G. K. OSTRANDER. 1987. *J. Biol. Chem.* 262: 15649-15658.
19. YAMASHITA, K., Y. TACHIBANA, T. OHKURA & A. KOBATA. 1985. *J. Biol. Chem.* 260: 3963-3969.
20. SHOREIBAH, M., G. S. PERNG, B. ADLER, J. WENSTEIN, R. BASU, R. CUPPLES, D. WEN, J. K. BROWNE, P. BUCKHALTS, N. FREGIEN & M. PIERCE. 1993. *J. Biol. Chem.* 268: 15381-15385.



# Genetic engineering of recombinant glycoproteins and the glycosylation pathway in mammalian host cells

Eckart Grabenhorst, Peter Schlenke, Susanne Pohl, Manfred Nimtz, and Harald S. Conradt\*

*Protein Glycosylation, GBF – Gesellschaft für Biotechnologische Forschung mbH, Mascheroder Weg 1, D-38124, Braunschweig, Germany*

The analysis of many natural glycoproteins and their recombinant counterparts from mammalian hosts has revealed that the basic oligosaccharide structures and the site occupancy of glycosylated polypeptides are primarily dictated by the protein conformation.

The equipment of many frequently used host cells (e.g. BHK-21 and CHO-cells) with glycosyltransferases, nucleotide-sugar synthases and transporters appears to be sufficient to guarantee complex-type glycosylation of recombinant proteins with a high degree of terminal  $\alpha$ 2-3 sialylation even under high expression conditions. Some human tissue-specific terminal carbohydrate motifs are not synthesized by these cells since they lack the proper sugar-transferring enzymes (e.g.  $\alpha$ 1-3/4 fucosyltransferases,  $\alpha$ 2-6 sialyltransferases). Glycosylation engineering of these hosts by stable transfection with genes encoding terminal human glycosyltransferases allows to obtain products with tailored (human tissue-specific) glycosylation in high yields.

Using site-directed mutagenesis, unglycosylated polypeptides can be successfully converted in N- and/or O-glycoproteins by transferring glycosylation domains (consisting of 7-17 amino acids) from donor glycoproteins to different loop regions of acceptor proteins.

The genetic engineering of glycoproteins and of host cell lines are considered to provide a versatile tool to obtain therapeutic glyco-products with novel/improved in-vivo properties, e.g. by introduction of specific tissue-targeting signals by a rational design of terminal glycosylation motifs.

**Keywords:** glycosylation engineering, human fucosyltransferases, human sialyltransferases, N- and O-glycosylation, host cell specificity, recombinant glycoprotein expression

## Introduction

The importance of the posttranslational modification of polypeptides with *N*- or *O*-linked oligosaccharides is well documented by their implication in numerous biological phenomena [1]. Consequently, it has already been recognized in the early eighties [2] that only mammalian host cells meet the criteria for an appropriate biotechnological development of recombinant glycotherapeutics to be used in humans. This has led to the attractive new research area of the biotechnology of mammalian cells as factories for medicinal glycoproteins.

Protein-linked oligosaccharides control the intracellular and tissue targeting of polypeptides, their half-life *in vivo* and their dynamic interaction with other proteins inside the cells or in body fluids. Carbohydrate structures of gly-

coproteins are typically polypeptide-specific and it has been shown that each individual glycosylation site of a glycoprotein may contain its own characteristic pattern of oligosaccharide chains [3]. Apart from the 3D-domain structure that governs its decoration with glycans, also the tissue or cell type that synthesizes a glycoprotein plays an important role in the phenomenon of microheterogeneity of protein glycans. This is a result of the regulated expression of a characteristic set of glycosidase and terminal glycosyltransferase genes which is different in the various cells/tissues of an organism [4] and may also vary with the physiological conditions of an organism or the differentiation state of cells. For example, human transferrin secreted from liver cells into the blood stream contains oligosaccharides usually found on serum glycoproteins, mostly afuco diantennary oligosaccharides with terminal  $\alpha$ 2,6-linked NeuAc, whereas the same protein isolated from human cerebrospinal fluid carries asialo and asialo-agalacto diantennary forms of proximally fucosy-

\*To whom correspondence should be addressed. Tel: +49-531-6181-287; Fax: +49-531-6181-202; E-mail: hco@gbf.de

lated chains with bisecting GlcNAc typical for glycoproteins synthesized in brain tissues [5-7] due to the different expression levels of the pertinent terminal glycosyltransferases.

Concepts for the biotechnological production of recombinant glycoprotein therapeutics or recombinant retrovirus vectors and *ex vivo* expansion of human primary cells for medicinal treatment must take into consideration different interactions of differently glycosylated cell/virus surface glycoconjugates or soluble glycotherapeutics with cellular receptors and subsequent altered modulation of intracellular signalling cascades. While during the past 12 years much work has been published on the structural characterization of recombinant glycoproteins expressed from various mammalian and nonmammalian expression systems, a great deal of efforts is presently going into attempts to improve recombinant host cell lines, and here especially mammalian cells, for the manufacturing of glycoprotein pharmaceuticals and retrovirus vectors with novel *in vivo* properties. It seems promising to explore the advantages of new generations of products with improved *in vivo* stability and carbohydrate-based tissue-targetable addressing signals. For this, the host cell lines must be improved by genetic engineering with newly introduced glycosyltransferases. The transferases must be stably directed into the proper subcellular compartment for their efficient function in the glycosylation pathway of the host. Here we are reporting on some of these aspects of the work from our laboratory at the German Center for Biotechnology (GBF) during the past 10 years.

## I. Recombinant expression of human therapeutic glycoproteins

### Mammalian host cells

Recombinant mammalian host cells cultured in large bioreactor systems are currently used to generate human glycoprotein pharmaceuticals which can be obtained from natural sources in only minute quantities. Amongst the first recombinant pharmaceutical glycoproteins produced from mammalian host cells were the secretory polypeptides interleukin-2 (IL-2), interferon- $\beta$  (IFN- $\beta$ ) and interferon- $\gamma$  (IFN- $\gamma$ ) [8-10]. Over the past 12 years the literature reporting on the glycosylation analysis of recombinant glycoproteins from different hosts has accumulated tremendously. In most of these studies Chinese hamster ovary (CHO) and baby hamster kidney cells (BHK-21) have been used as expression systems and most of our present knowledge about the culture conditions that can affect the fine structural characteristics of recombinant glycoproteins produced in large scale processes has been obtained from studies with these two host cells [11-14]. In our hands, a constitutively secreted glycoprotein expressed at a level of 0.1  $\mu\text{g}/\text{ml}$  from BHK-21 or CHO cells has the same carbo-

hydrate structure as has the protein expressed at a 200-fold higher level, and as a general rule, it appears that the glycosylation machinery of the host cell itself is not a bottleneck for an efficient posttranslational modification of a polypeptide with carbohydrates. However, problems might eventually be encountered when a recombinantly expressed protein has an abnormal half-life in different cellular compartments of the host. It should be noted that in rare cases the selection procedures used for the isolation of transfected high expression cell clones may lead to the detection of a variant cell clone with aberrant glycosylation capacity, as has been recognized in our laboratory with a BHK-21 cell line that showed a complete loss of carrying out complex-type glycosylation of a recombinantly expressed protein [15].

As has become clear from the work of others and our own investigations, CHO and BHK-21 cells show basically the same characteristics for the glycosylation of recombinant *N*- or *O*-glycoproteins. The structural features detected in recombinant glycoproteins expressed from murine and hamster cell lines are summarized in Table 1. In principle, the antennarity and the LacNAc content of *N*-linked oligosaccharides of a given recombinant glycoprotein expressed in CHO, BHK-21 or the murine cell lines will be the same and this is also true for characteristics of

**Table 1.** Structural features of *N*-linked oligosaccharides from recombinant glycoproteins expressed in mammalian host cells. Data are based on structural analysis of the recombinant human glycoproteins IFN- $\beta$ , Epo, AT III, IL-6, tissue-plasminogen activator and  $\beta$ -TP as well as recombinant humanized antibodies, soluble receptor proteins and *N*-glycosylation mutants of human IL-2.

carbohydrate structure	host cell line			
	CHO	BHK-21	C127	Ltk <sup>-</sup>
proximal fucose	+	+	+	+
Fuc( $\alpha$ 1-2)Gal-R*	+	+	?	?
$\alpha$ 2,6-NeuAc	-	-	+	+
$\alpha$ 2,3-NeuAc	+	+	+	+
NeuAc( $\alpha$ 2-8)NeuAca2-3-R	+	+	-	+
NeuGly*	+	+/-	+	+
tri/tetra-antennarity	+	+	+	+
Gal( $\beta$ 1-4)GlcNAc repeats	+	+	+	+
Gal( $\beta$ 1-3)GlcNAc-R	-	+	-	-
sulfated glycans	+	+	+	+
Gal( $\alpha$ 1-3)Gal	+	-	+	+
branched repeats	?	-	+	-
mannose 6-phosphate*	+	+	?	?
bisecting GlcNAc	-	-	+	+
GalNAc( $\beta$ 1-4)GlcNAc	-	+**	-	-

\*detectable only in trace amounts

\*\*detected in large amounts in the BHK-21A variant cell line [16,17]

the oligosaccharide pattern at individual glycosylation sites. However, in view of the pronounced higher microheterogeneity of terminal carbohydrate motifs in recombinant products obtained from the murine host cells ( $\alpha$ 2,3- vs.  $\alpha$ 2,6-NeuAc, NeuGly, Gal( $\alpha$ 1 $\rightarrow$ 3)Gal, Gal( $\beta$ 1 $\rightarrow$ 3)GlcNAc-R, sulfated structures and branched repeats in Ltk<sup>-</sup> and C127 cells), the two hamster cell lines seem to provide a more favourable expression host cell system when low glycoform heterogeneity is required.

It should be emphasized that in most publications on carbohydrate structures of recombinant glycoproteins the work has been performed with purified glycoprotein preparations destined for pharmaceutical use. Consequently, these preparations represent a subfraction of the total product secreted by the host cell and they are enriched in glycoforms which are believed to be most effective for *in vivo* application in humans. One such example is recombinant human erythropoietin (EPO) from BHK-21 or CHO cells where only a highly sialylated subfraction (based on the isoelectric focussing pattern) of the total recombinant glycoprotein hormone that is secreted by the producer cells is manufactured for medical treatment. This subfraction represents only about 20–25% of the total EPO secreted from the host cell lines. Therefore, for a complete description of the glycosylation characteristics of any host cell, it is indispensable to purify the product quantitatively, e.g., by immunoaffinity chromatography using polypeptide-specific antibodies that guarantee >90% final yield.

Significant advances in the sensitivity of carbohydrate structural analysis has been achieved during the past three years. Especially in mass spectrometry (on-line ESI-MS, nanospray tandem mass spectrometry (ESI-MS/MS) and improved MALDI/TOF techniques), very sensitive instrumentation for glycosylation analysis has been made available to a broader group of research units, and thus has led to a broader use of complementary tools by academic researchers and in industrial laboratories. This is of outstanding importance in the area of glycobiology and glycotechnology where the combined methods of molecular biology, protein biochemistry, cell biology and analytical know-how are required to understand in detail the basic mechanisms and the role of modification of proteins and lipids with carbohydrate in both, health and different states of disease.

#### Production of secretory glycoproteins in insect cells using the recombinant baculovirus expression system

Some 10 years ago it has been proposed to use insect cells infected with recombinant baculoviruses for production of large amounts of recombinant glycoproteins. However, it has become clear that the insect expression system has its limitations for the production of mammalian-type

modified glycotherapeutics. We found that secretory glycoproteins that contain complex-type *N*-glycans when expressed in mammalian host cell lines are modified only with the short oligomannosidic Man( $\alpha$ 1 $\rightarrow$ 6)Man( $\beta$ 1 $\rightarrow$ 4) GlcNAc( $\beta$ 1 $\rightarrow$ 4)[Fuc( $\alpha$ 1 $\rightarrow$ 6)]GlcNAc and Man( $\alpha$ 1 $\rightarrow$ 3)[Man( $\alpha$ 1 $\rightarrow$ 6)]Man( $\beta$ 1 $\rightarrow$ 4) GlcNAc( $\beta$ 1 $\rightarrow$ 4)[Fuc( $\alpha$ 1 $\rightarrow$ 6)]GlcNAc *N*-glycans when expressed from Sf21 or Sf9 (*Spodoptera frugiperda*) cells [18]. *N*-glycan structures most similar to those synthesized in Sf9 or Sf21 cells were also detected in the products secreted from SPC-Bm36 (*Bombyx mori*) cells (see Table 2). SPC-Bm36 cells produce *N*-glycosylated proteins with higher amounts of dimannosyl- over trimannosyl-oligosaccharides and only 60%  $\alpha$ 1,6-fucosylation of the proximal GlcNAc. A model glycoprotein with a potential *O*-glycosylation motif [18] expressed from SPC-Bm36 cells was found to be unglycosylated, modified with GalNAc or with Gal( $\beta$ 1 $\rightarrow$ 3)GalNAc in a ratio of 1:3:5 that is different in the same protein when synthesized from Sf21 cells, where a ratio of 1:4:4 was detected [18]. Therefore, it appears that SPC-Bm36 cells generally underglycosylate *N*- and *O*-glycoproteins.

*N*-glycan structures of glycoproteins expressed from BTI-Tn-5B1-4, "High Five" (*Trichoplusia ni*) cells are essentially the same as those from Sf21 cells and are present in a similar ratio. However, a considerable proportion of the oligosaccharides was found to be *difucosylated*, containing an additional fucose in  $\alpha$ 1,3-linkage to the proximal GlcNAc. The enzymatic activity involved in the biosynthesis of this structural motif has also been described for a *Mamestra brassica* cell line, IZD Mb0503 [19]. Surprisingly, the analysis of a *N*-glycosylated protein variant expressed in BTI-EaA (*Estigmene acrea*) cells revealed the presence of fucosylated trimannosyl-oligosaccharides containing 1,2, or small amounts of even 3 terminal GlcNAc-residues as detected by methylation analysis and ESI-MS/MS of the pertinent tryptic glycopeptide [20]. However, no indication for galactosylated oligosaccharides was detected by the complementary analytical techniques applied in our laboratory. Such complex-type *N*-glycan structures were not detected on recombinant glycoproteins expressed in any other baculovirus-infected insect cell line shown in Table 2. These data clearly confirm our previous results [18] and those of others [21,22] that insect cell lines are incapable of synthesizing sialylated lactosamine complex-type *N*-glycans or sialylated core 1 *O*-glycans and therefore are not suitable for the production of recombinant pharmaceutical glycoproteins for clinical use.

An further disadvantage of the insect cell expression system is that recombinant baculovirus vector-driven high expression of proteins is run as a batch-culture process and cells die after infection within the productive phase. Only about 20% of the total recombinant protein synthesized by the host cells is secreted into the supernatant. The remainder is found denatured as inclusion body-like aggregates

**Table 2.** Glycosylation characteristics of recombinant glycoproteins secreted from 6 different lepidopteran cell lines. Data were obtained by detailed carbohydrate analysis of human  $\beta$ -TP, IFN- $\beta$  and different human IL-2 N-glycosylation variants secreted from baculovirus-infected cells. Cell cultures were run in tissue culture flasks and in bioreactors, and the products were analysed from harvests after 2 days, 4 days, and 7 days post infection. M = mannose, Gn = N-acetylglucosamine, F = fucose

Insect cell line	N-glycan structures of secreted recombinant glycoproteins											
	M	M	M	F	M	F	M	F	M	F	Gn	
M—Gn—Gn	\	\	\	16	/ \	16	/ \	16	/ \	16	/ \	F
M—Gn—Gn	/	M—Gn—Gn	M—Gn—Gn	/	M—Gn—Gn	/	M—Gn—Gn	/	M—Gn—Gn	/	M—Gn—Gn	16
M	M	M	M	M	M	M	M	M	M	M	M	M—Gn—Gn
Spodoptera frugiperda (Sf21)	+	+	+++	+++	+	—	—	—	—	—	—	—
Spodoptera )	+	+	+++	+++	+	—	—	—	—	—	—	—
frugiperda (Sf9	+	+	++	++	—	—	—	—	—	—	—	—
Trichoplusia ni (BTI Tn5B1-4, "High-Five")	++	++	++	++	—	++	++	++	—	—	—	—
Bombyx mori (SPC-Bm36)	++	+	++	+	—	—	—	—	—	—	—	—
Mamestra brassicae (IZD Mb0503)	—	—	++	++	—	+++	+++	++	—	—	—	—
Estigmene acrea (BTI-EaA)	—	—	++	++	—	—	—	++	++	++	++	+

inside the cell and is partially released into the medium at later stages of infection from dying cells [23]. Nevertheless, the baculovirus expression system is the preferred system when the production of several 100 mg of a protein is required for research purposes within a short time and a mammalian/human-type of glycosylation is not of primary importance.

## II. Genetic engineering of new glycoproteins and their recombinant expression in animal cells

### Engineering of proteins with new glycosylation properties

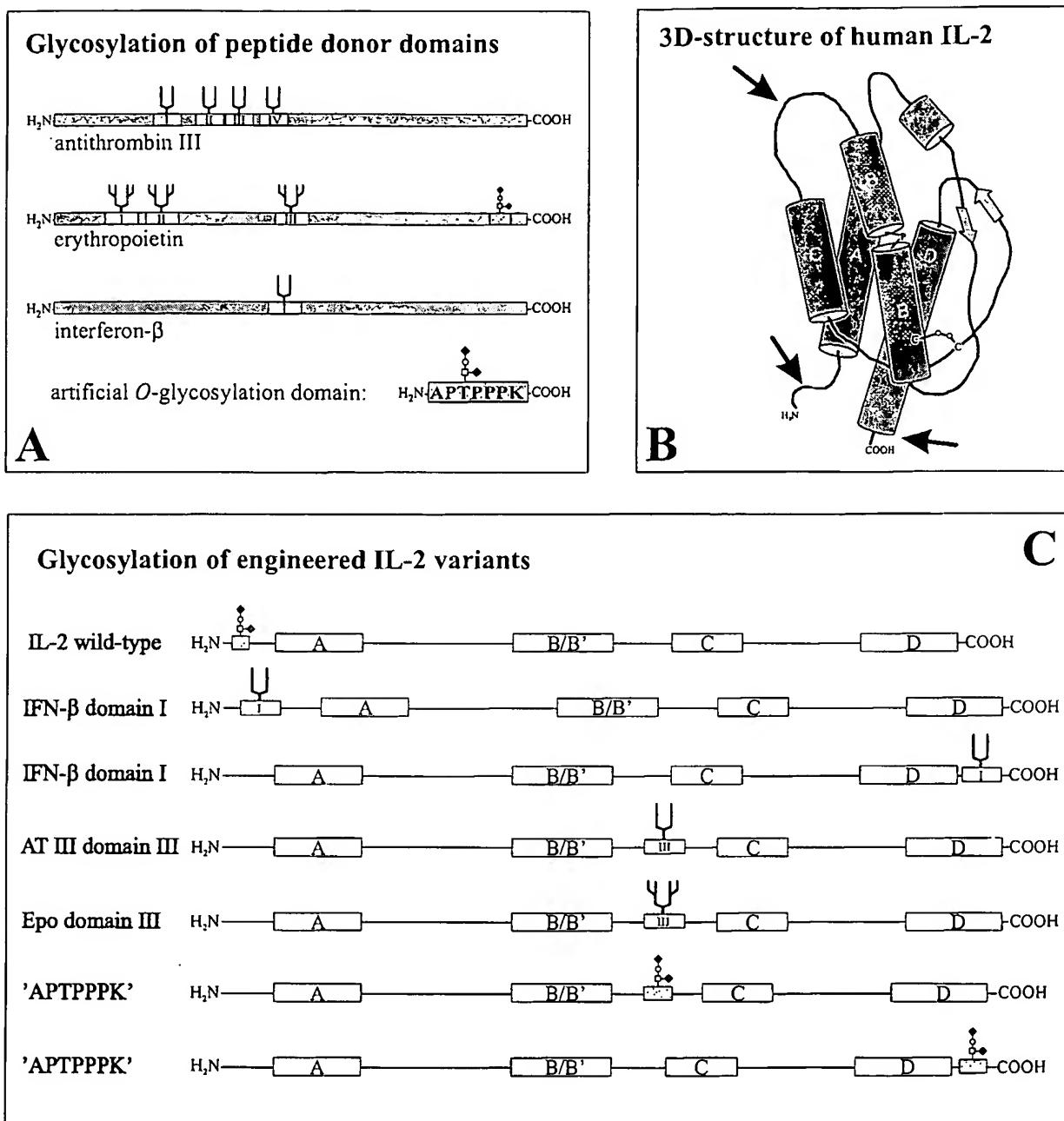
The successful modification of a polypeptide with newly introduced *N*- or *O*-glycosylation properties may increase its solubility or influence its *in vivo* biological properties (e.g., activity, antigenicity, rate of clearance). In other cases, the simple deletion of a glycosylation site with pronounced carbohydrate microheterogeneity might also be of advantage for a final clinical application of the product. It is now known from the 3D-structures of many glycoproteins that *N*- or *O*-glycosylation motifs are mostly found in loop-regions of polypeptides. According to our experience, a single amino acid exchange creating a new potential consensus tripeptide Asn-Xxx-Ser/Thr is often not sufficient for *N*-glycosylation to occur, even when present in loop regions. The concept of polypeptide-specific and glycosylation site-specific modification of proteins with carbohydrates has led us to investigate by using site-directed mutagenesis the introduction of individual *glycosylation domains* from donor glycoproteins with known glycosylation characteristics into suitable locations of model acceptor proteins (IL-2, IFN- $\beta$ ). This approach aimed at the definition of short peptide domains that should result in predictable oligosaccharide structures when the constructs are expressed from a given host cell. The insertion of short (8–15 residues) peptide sequences containing an Asn-Xxx-Thr/Ser sequence has been proved to be successful, and, most importantly, was found not to severely affect the overall 3D-structure as shown for the biological activity of the resulting human IL-2 and IFN- $\beta$  variants [23,24]. We have analyzed the carbohydrate structure of the different resulting chimeras after expression from BHK-21 cells as shown in Figure 1. The single *N*-glycosylation site of human IFN- $\beta$  contains preponderantly diantennary complex-type oligosaccharide chains when expressed from CHO or BHK-21 cells [9,25], as is the case for all four *N*-glycosylation sites of human antithrombin III (AT III) [26]. The three *N*-glycosylation sites of human EPO from the same host cells contain preponderantly tetraantennary chains with 1-3 *N*-acetyllactosamine repeats [27–30], with Asn<sub>86</sub> (site III) bearing the most homogenous oligosaccharide population. As mentioned above, the introduction of a new *N*-glycosylation site into the IL-2 polypeptide by substitu-

tion of Thr<sub>3</sub>→Asn (APNSSSTKKT<sub>10..</sub>) does not result in any modification with *N*-glycans when the construct is expressed from BHK-21 or *Ltk*<sup>−</sup> cells [24]. However, the transfer of the human IFN- $\beta$  *N-glycosylation domain* SSSTGWNETIV(GG) to the N- or C-terminus of IL-2 yielded proper *N*-glycosylation with diantennary complex-type chains [31,32], as is the case for wild-type IFN- $\beta$  from these hosts [9,25].

Similarly, the peptide comprising the *N*-glycosylation domain III of human AT III was found to be modified with diantennary *N*-glycans when inserted at position 80 within the loop region between helices B' and C of human IL-2 as is depicted in Figure 1. However, the introduction of *N*-glycosylation domain III of human EPO at the same location resulted in oligosaccharides with significantly higher antennarity. These investigations indicate that *N*-glycosylation domains can successfully be transferred from one protein to a loop region or the N- or C-terminus of another protein. Our data allow for the conclusion that in several cases the characteristic antennarity of the donor *N*-glycosylation domain is preserved when inserted into the newly constructed mutant glycoprotein [11,23,24,31,32].

### Engineering of O-glycosylated proteins

All mammalian cell lines frequently used for recombinant protein expression (e.g. the cell lines in Table 1) modify *O*-glycosylation sites with preponderantly core 1 *O*-glycans containing one or two NeuAc [27,29,33]. In secretory glycoproteins like human IL-2 or EPO, which are *O*-glycosylated at a single hydroxyamino acid, all recombinant host cells recognize specifically the same Ser or Thr that is modified in the natural protein even when it is part of a hydroxyamino acid cluster [11,24], as is the case for human IL-2 where specifically Thr<sub>3</sub> within the N-terminal sequence H<sub>2</sub>N-APT<sub>3</sub>SSSTKKT<sub>10..</sub> is modified by NeuAc(α2→3)Gal(β1→3)[NeuAc(α2→6)]<sub>0-1</sub>GalNAc chains [33]. As is summarized in Table 3, mutant IL-2 proteins with substitution of Thr<sub>3</sub>→Ser or containing Thr at position 5 are not *O*-glycosylated [24]. The deletion of Thr<sub>3</sub> or its substitution with Ala or Ser abolishes *O*-glycosylation completely, whereas the exchange of Ser<sub>4</sub> or Ser<sub>6</sub> to Thr results in detectable *O*-glycosylation. The peptide sequences APTPP, APTAPPT (present in human plasminogen) or the artificial sequence APTPPP can be used to introduce novel *O*-glycosylation sites into human IL-2 or human IFN- $\beta$  at different positions of the polypeptide chain [11,18,24,32], and the resulting proteins are efficiently *O*-glycosylated in BHK-21 cells and *Ltk*<sup>−</sup> cells, as is indicated also in Figure 1. Thus, these sequence motifs can be considered to constitute general transferable *O*-glycosylation recognition domains when introduced into loop regions of polypeptides although no consensus sequence for the attachment of *O*-glycans to polypeptides has yet been identified.



**Figure 1.** Genetic engineering of glycoproteins with defined glycosylation characteristics: Insertion of *N*- and *O*-glycosylation domains into human IL-2. *panel A*, glycosylation domain donor glycoproteins with known glycosylation characteristic; *panel B*, 3D-structural model of human IL-2 based on NMR data; arrows indicate acceptor sites used for insertion of glycosylation domains; *panel C*, glycosylation acceptor characteristics of newly introduced domains as identified by analysis of the resulting IL-2 variants. Bars A, B/B', C and D indicate helical domains of human IL-2 as shown in Figure 1B

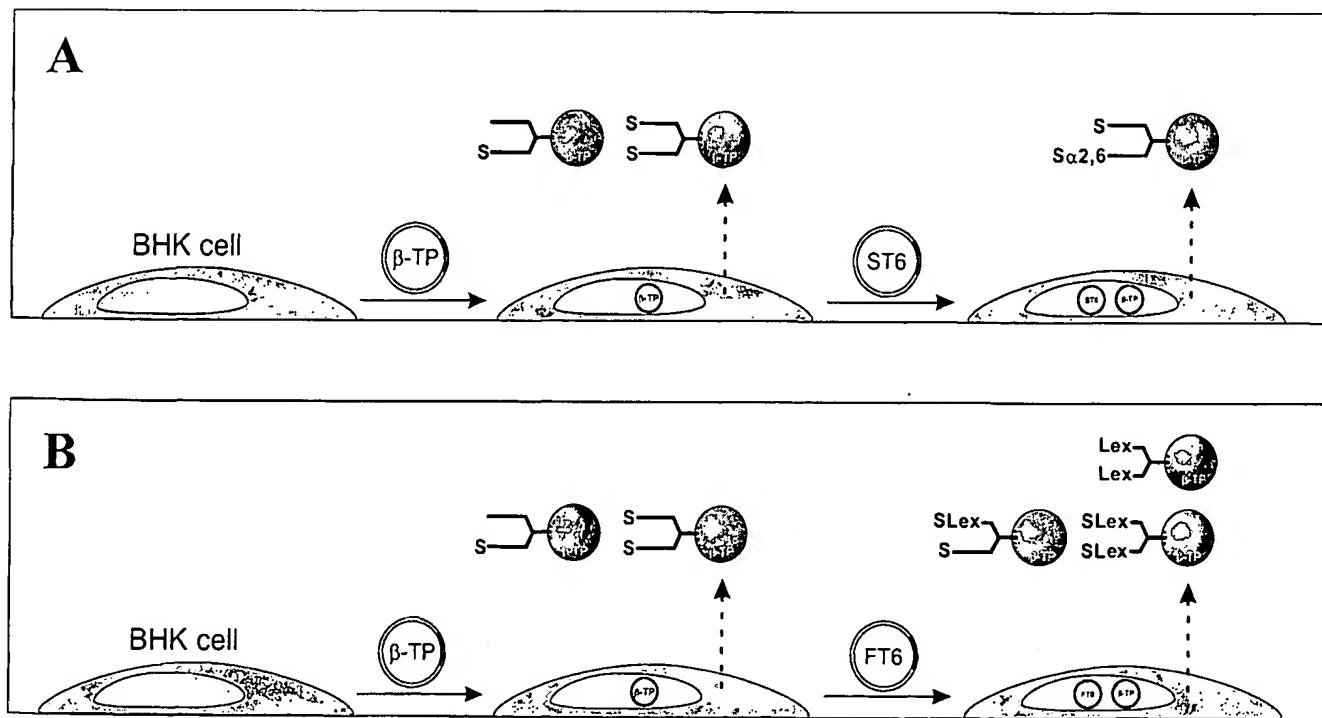
**Table 3.** Mutation analysis of the O-glycosylation acceptor properties of the N-terminus of human IL-2. Data were corroborated by immunoprecipitation of stably (BHK-21) as well as transiently (*Ltk*<sup>-</sup>) transfected mammalian host cells and N-terminal sequencing of the purified proteins.

N-terminal sequence	O-glycan attached at position
AP <u>T</u> SSSTKKT ...	3
AP <u>A</u> SSSTKKT ...	no
AP <u>S</u> SSSTKKT ...	no
AP <u>S</u> SS <u>T</u> KKT ...	no
APT <u>I</u> SSSTKKT ...	3↑
AP <u>T</u> SS <u>I</u> TKKT ...	3↓
AP <u>T</u> SS <u>I</u> TKK <u>T</u> ...	3↑
AP <u>N</u> SSSTKKT ...	no
AP <u>S</u> TSSTKKT ...	no
AP <u>S</u> SI <u>T</u> KKT ...	no
AP <u>S</u> SS <u>I</u> TKKT ...	no
APT <u>A</u> PPTKKT ...	3↑, 7
APT <u>P</u> PPSTKKT ...	3↑
APT <u>P</u> PP <u>T</u> KKT ...	3↑

### III. Construction of host cell lines with novel glycosylation characteristics

#### In vivo specificity of glycosyltransferases

*In vitro* assays of glycosyltransferases with small acceptor substrates may yield some preliminary information about acceptor substrates properties recognized by the enzymes and are indispensable for the evaluation of glycosyltransferase levels in cells/tissues and the control of enzyme purification. A final description and comparison of the *in vivo* specificity of the individual glycosyltransferases, however, can only be achieved by structural analysis of the cellular product(s). As shown in Figure 2, we suggest the recombinant expression of the full length form of human glycosyltransferases along with a suitable reporter glycoprotein (here human  $\beta$ -TP) at a constant expression level in a heterologous mammalian host cell line that is devoid of the pertinent enzyme activity. This is considered to represent a valuable model and should enable the comparison of the *in vivo* specificities of different members of a glycosyltransferase family [16,17,34,35] and allow the selection of the optimal enzyme suitable for the glycosylation engineering of host cell lines for the production of a new generation of glycotherapeutics with defined altered glycosylation characteristics. Basic information can be expected from such



**Figure 2.** Genetic engineering of new BHK host cells by transfection with human glycosyltransferase genes. In order to generate expression of a reporter glycoprotein, BHK-21 cells were transfected with a plasmid encoding human  $\beta$ -TP. The recombinant  $\beta$ -TP secreted from such cell lines is glycosylated host cell-type-specifically with  $\alpha$ 2,3-di- or monosialo diantennary complex-type *N*-glycans (with proximal fucose) as indicated by "S" attached to the structural symbols. *panel A*, cotransfection with the human ST6Gal I gene leads to secretion of  $\beta$ -TP containing  $\alpha$ 2,6-linked NeuAc; *panel B*, cotransfection with human  $\alpha$ 1,3-fucosyltransferase VI (FT6) results in  $\beta$ -TP modified with sLex- or Lex-containing oligosaccharides

studies concerning the intracellular organization of the protein glycosylation machinery and the temporal and spatial distribution of the transferases in the *in vivo* biosynthetic compartments. Prerequisites for such an *in vivo* assay system are:

- i. Reproducible transfection procedures using high expression vectors and rapid selection/isolation of stably transfected cells
- ii. A constant level of acceptor substrate expression (reporter glycoconjugate) by the host cells
- iii. A defined expression level of the recombinant enzymes (20- to 50-fold higher expression levels of the wild-type forms are achieved in transfected cells when compared to the levels in primary cells/tissues)
- iv. A simple, fast and quantitative purification procedure for the product (preferably secreted into the medium)
- v. Application of fast and sensitive carbohydrate structural analytical micromethods (MS and MS/MS-techniques, HPAE-PAD)

It should be emphasized that the 3D-structure of the glycoprotein substrate and thus the accessibility of its oligosaccharide moieties under the intracellular environmental conditions is also of importance. In addition, it is conceivable that cell surface membrane glycoproteins might be recognized differently than are obligate secretory glycoproteins. However, for a given model glycoprotein with defined structural characteristics, the above approach by analysis of a reporter glycoconjugate from stably transfected cells should yield precise information on the *in vivo* substrate specificity of the individual members of a family of enzymes acting on the same precursor substrate. Transient expression experiments are of limited value, since cell damage and cell leakage resulting from the transfection procedures is considered to lead to artefacts.

#### Engineering of cells by stable transfection with human $\alpha$ 1,3/4-fucosyltransferases genes

*Analysis of the in vivo acceptor substrate specificity of fucosyltransferases by glycosylation analysis of coexpressed recombinant human  $\beta$ -trace protein.*

The  $\alpha$ 1,3/4-fucosyltransferases III-VII [36–41] add fucose to the GlcNAc residue in sialylated or unsialylated Gal( $\beta$ 1 $\rightarrow$ 3)GlcNAc-R or Gal( $\beta$ 1 $\rightarrow$ 4)GlcNAc-R type structures of glycoconjugates. They are thus involved in the regulation of the synthesis of the Lewis X (Lex) and sialyl Lewis X (sLex) type ligands that are involved in inflammation-induced adhesion of neutrophils, monocytes, T cells and platelets to selectins [42–45]. Fucosylated glycoconjugates play also a central role in other important biological phenomena like differentiation and tumorigenesis, and elevated levels of peripherally fucosylated serum glycopro-

teins have been detected in humans associated with inflammatory processes [46,47].

Many natural human tissues/cells express more than one fucosyltransferase at the same time and therefore it is difficult to obtain homogenous enzyme preparations from natural tissues or body fluids for the unequivocal assessment of the specificity of the individual enzymes. The cloning of the  $\alpha$ 1,3/4-fucosyltransferases III-VII (FT3-FT7) and their expression in recombinant form has provided a tool to isolate pure enzyme preparations for studying their substrate specificity *in vitro*. However, several questions concerning the implication of each of the individual fucosyltransferases in the generation of selectin ligands are still not resolved.

Mammalian fucosyltransferases are Golgi-resident type II transmembrane proteins, and according to current opinion, their transmembrane region is responsible for the retention of the enzymes in the proper Golgi compartment. Many transferases contain N-glycosylation sites in their stem region and/or their catalytic domain; however, no information is available if, or to what extent, N-glycosylation is involved in the *in vivo* activity or specificity of fucosyltransferases. A number of publications have appeared that describe the recombinant expression of human fucosyltransferases [48–52] mostly as soluble forms lacking the cytoplasmic, the transmembrane and some part of the stem region. In several cases, recombinant chimeras containing N-terminally fused polypeptide fragments (*e.g.*, of protein A) have been constructed to facilitate recombinant enzyme purification. According to the data published so far, FT7 has been reported to fucosylate exclusively  $\alpha$ 2,3-sialylated N-acetyllactosamine-type structures *in vitro* and is inactive with neutral acceptors [41,51,52]. FT4 acts almost exclusively on unsialylated Gal( $\beta$ 1 $\rightarrow$ 4)GlcNAc-R (type II) structures [48,53], whereas *in vitro*, FT5 and FT6 have been reported to act on both,  $\alpha$ 2,3-sialylated as well as unsialylated type II acceptors [48,49,54]. FT3 has been reported to mainly transfer Fuc in  $\alpha$ 1,4-linkage onto GlcNAc in type I chains [34,48,54]. Activity with type I acceptors has also been found for human FT5 [48], while FT4, FT6 and FT7 are not active with Gal( $\beta$ 1 $\rightarrow$ 3)GlcNAc-R substrates [41,48,54].

Human  $\beta$ -TP is a 168 amino acid protein which contains two N-glycosylation sites that are occupied with almost exclusively diantennary complex-type chains [5,16,35,55]. Similar to human transferrin described above,  $\beta$ -TP isolated from human cerebrospinal fluid exhibits "brain-type" glycosylation characteristics, *i.e.*, mainly truncated asialo chains, bisecting GlcNAc, complete proximal and some peripheral fucosylation besides small amounts of  $\alpha$ 2,3/6-sialylated N-glycans [5,7]. Recombinant human  $\beta$ -TP expressed from wild-type BHK-21B cells is also modified with almost exclusively diantennary oligosaccharides at each of its two N-glycosylation sites, however, as shown in Figure 3, the oligosaccharide pattern here is very homogenous, the structures contain either two or one  $\alpha$ 2,3-linked NeuAc and only small amounts of asialo chains are present [16,35].

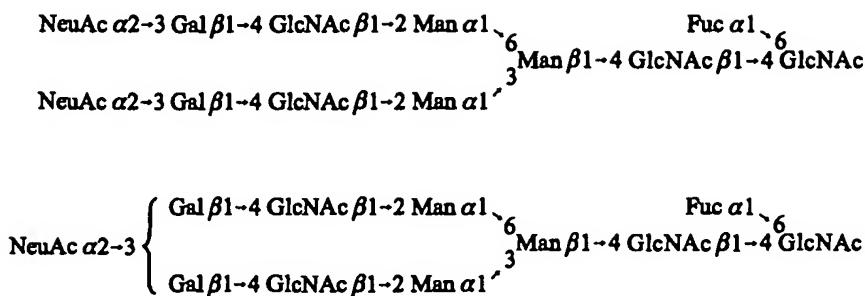


Figure 3. *N*-glycan structures of recombinant human  $\beta$ -TP secreted from wild-type BHK-21 cells contain exclusively  $\alpha$ 2,3-linked NeuAc.

Coexpression of  $\beta$ -TP as a reporter glycoprotein from cells transfected with a human  $\alpha$ 1,3/4-fucosyltransferase therefore should yield oligosaccharides with Lewis X or sialyl Lewis X motifs or mixtures of the two motifs depending on the *in vivo* specificity of the transfected fucosyltransferase gene. In total 12 different diantennary *N*-linked oligosaccharides can be expected in  $\beta$ -TP from BHK-21 cells expressing human FT6 (four each of asialo, mono- and disialo chains with no, one or two peripheral fucose residues, respectively).

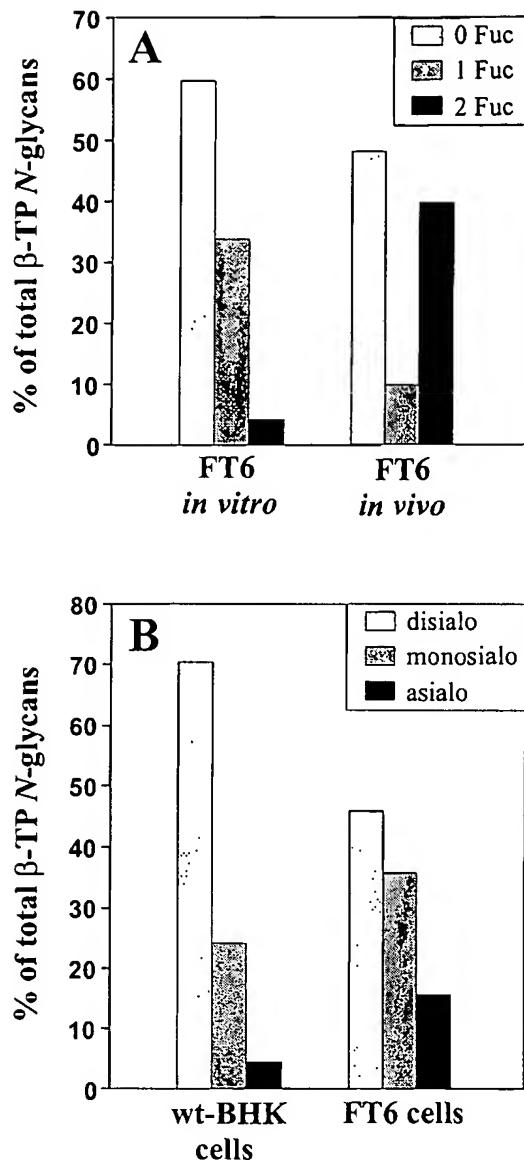
Human FT6 synthesizes preponderantly  $\alpha$ 1,3-difucosylated structures on diantennary chains *in vivo*

When incubated *in vitro* with soluble FT6 in the presence of GDP-Fuc, we found efficient fucosylation of  $\beta$ -TP, with

a roughly 50% modification of the  $\alpha$ 2,3-monosialylated oligosaccharide with one  $\alpha$ 1,3-linked Fuc, and with 33% and 6% modification of the  $\alpha$ 2,3-disialylated oligosaccharides with one or two  $\alpha$ 1,3-linked Fuc residues, respectively [35]. This result obtained for the complex type *N*-glycans is in agreement with published reports for the *in vitro* specificity of recombinant human FT6, which indicate that the enzyme can form Lex as well as sLex motifs with small type II oligosaccharides [49,54]. When  $\beta$ -TP is coexpressed from BHK-21 cells together with human FT6 (see Table 4), about 50% of all *N*-glycans contain  $\alpha$ 1,3-linked Fuc (*cf.* Fig. 4A). However, the sialylation degree of the *N*-glycans is significantly lower when compared to *N*-glycans of  $\beta$ -TP from wild-type BHK cells (Fig. 4B), and also in contrast to the *in vitro* modified  $\beta$ -TP, most of the oligosaccharides are found to be modified *in vivo* with two peripheral Fuc (see

Table 4. Fucosyltransferase activities of stably transfected BHK-21 cell lines. Measurements were performed using the substrate GDP-[<sup>14</sup>C]Fuc and Gal(β1→4)GlcNAc-O-(CH<sub>2</sub>)<sub>8</sub>-COOCH<sub>3</sub> as an acceptor. A dash indicates incorporation of radioactivity at background levels. \*FT3 activity was detected by using the type I Gal(β1→3)GlcNAc-O-(CH<sub>2</sub>)<sub>8</sub>-COOCH<sub>3</sub> acceptor; \*\*FT7 was determined with native bovine fetuin. *In vitro* activity values for FT5 and FT7 are very low; however, from standard transfection procedures used, and in view of the *in vivo* fucosylation efficiency (see Fig. 5), an expression value similar to those detected for the other fucosyltransferases is assumed.

Cell line	Total activity (cells + culture medium) $\mu$ U $\times$ 10 <sup>-6</sup> cells $\times$ 48 h <sup>-1</sup>	% of total activity accumulated in the culture medium after 48 hours
BHK-21B (wild-type)	—	—
FT3	—	—
FT3*	100	78%
FT4	75	27%
FT5	1	0%
FT6 (BHK21-B)	170	74%
FT6 (BHK-21A)	210	88%
FT6 (CHO DHFR-)	370	81%
s-FT6(I)	175	92%
s-FT6(II)	2970	91%
BT-FT6	9400	96%
FT7	—	—
FT7**	3	0%



**Figure 4.** Comparison of  $\beta$ -TP oligosaccharides after *in vitro* or *in vivo* modification with human FT6. For *in vitro* fucosylation, purified  $\beta$ -TP expressed from BHK-21 cells was incubated with recombinant soluble human FT6 (s-FT6) in the presence of GDP-Fuc. *In vivo* data were obtained following coexpression of  $\beta$ -TP and full-length FT6 genes in BHK-21 cells (*cf.*, Fig. 2). *panel A*, percentage of nonfucosylated,  $\alpha$ 1,3-mono- and  $\alpha$ 1,3-difucosylated N-glycans; *panel B*, percentage of disialo, monosialo and asialo diantennary chains in  $\beta$ -TP N-glycans

**Table 5.** In vivo fucosylation characteristics of human  $\alpha$ 1,3/4-fucosyltransferases. Values represent the percentage of all dianhydronary oligosaccharide forms comprising >90% of total N-glycans isolated from  $\beta$ -TP expressed in each individual FT-transfected BHK-21 cell line. Structural analysis was performed by HPAE-PAD mapping, MALDI/TOF-MS, ESI-MS/MS and methylation analysis. The two isomeric monosialo/monofucosylated structures from FT3, FT5 and FT6 cells were not resolved.

BHK cell line	$\beta$ -TP N-glycan structures (%)									
wild-type	75	20	5	—	—	—	—	—	—	—
FT3	59	20	2	3	13	—	—	3	—	—
FT4	40	9	—	—	5	4	—	21	17	3
FT5	46	18	2	8	12	5	—	7	—	2
FT6	29	16	3	13	5	15	—	5	—	13
FT7	43	22	4	11	11	—	9	—	—	—
s-FT6(I)	59	35	6	—	—	—	—	—	—	—
BT-FT6	55	17	2	5	7	6	—	3	—	4
										1

also Table 5). The ratio of sLex:Lex antennae in the total N-glycan mixture is 1.1:1. The lower sialylation degree of  $\beta$ -TP from FT6 cells compared to wild-type BHK-21 cells can be explained by an *in vivo* competition of the recombinantly expressed FT6 with the endogenous  $\alpha$ 2,3-ST(s), ST3Gal III and/or ST3Gal IV, for the common asialo oligosaccharide substrate. This phenomenon has been observed previously for products from recombinant host cell lines transfected with  $\alpha$ 1,3-galactosyltransferase [56] or FT4 [57]. The  $\alpha$ 2,3-STs have been reported to be unable to sialylate Gal( $\beta$ 1 $\rightarrow$ 4)[Fuc( $\alpha$ 1 $\rightarrow$ 3)]GlcNAc-R motifs [41,50,57]. Obviously, as is the case for *in vitro* incubation conditions, FT6 can act also *in vivo* on both,  $\alpha$ 2,3-sialylated as well as unsialylated N-linked oligosaccharides. To our knowledge, this is the first publication describing quantitatively and in detail the *in vivo* substrate specificity of a recombinant fucosyltransferase expressed in a stably transfected heterologous host cell line [35].

#### All human $\alpha$ 1,3/4-fucosyltransferases synthesize sLex structures *in vivo*

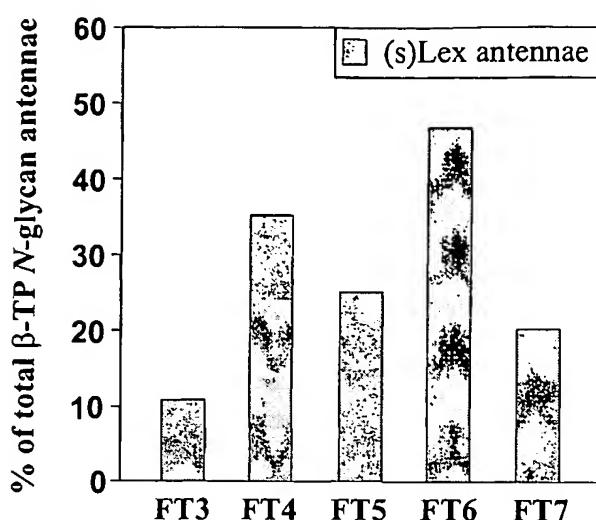
The differences of the *in vitro* and *in vivo* results obtained for FT6 led us to compare the *in vivo* properties of all human  $\alpha$ 1,3/4-fucosyltransferases. For this, we have also constructed stable BHK-21 cell lines (see Table 4) expressing human FT3, FT4, FT5 or FT7 together with human  $\beta$ -TP as a reporter glycoprotein, and for each individual cell line,  $\beta$ -TP was purified from the culture supernatant and subjected to oligosaccharide structural analysis using MALDI/TOF-MS, ESI-MS/MS and HPAE-PAD mapping. About 30–50% of the N-linked oligosaccharides of  $\beta$ -TP secreted from the new cell lines were  $\alpha$ 1,3-fucosylated except for the FT3 cell line in which case only 19% of the structures were fucosylated. A comparison of all  $\beta$ -TP N-

glycan structures formed by the  $\alpha$ 1,3/4-FT-transfected cells is presented in Table 5 and gives an overview of the *in vivo* substrate specificity of the five known human  $\alpha$ 1,3/4-FTs with protein-bound complex-type N-linked oligosaccharides [35].

**FT7 cells** (*i.e.*, BHK-21 cells coexpressing FT7 and  $\beta$ -TP) exclusively synthesize sLex structures. We have confirmed this also for the monosialylated oligosaccharide fraction that did not contain any  $\alpha$ 1,3-difucosylated structure. The single Fuc was exclusively present as the sLex and *not* as the Lex motif (determined by ESI-MS/MS, see, ref. [35]), and the small amount of asialo oligosaccharides did not contain any Lex epitopes. This *in vivo* specificity with complex-type N-glycans is in agreement with recent work published on the *in vitro* activity of the enzyme with small oligosaccharide substrates [41,50,51].

The vast majority of the peripherally fucosylated product in  $\beta$ -TP oligosaccharides from **FT4 cells** were found to contain the Lex motif which result is compatible with published data on the *in vitro* activity of FT4 with low molecular weight compounds. From the fragmentation pattern of reduced and permethylated chains using ESI-MS/MS, we could show that the monosialylated  $\alpha$ 1,3-monofucosylated N-glycan contains preponderantly the Lex motif [35]. However, a significant amount (11%) of mono-sLex was also observed in the disialo oligosaccharide fraction which contradicts published data on the *in vivo* specificity of the enzyme as measured by E-selectin binding studies [41,53,58,59]. However, no  $\alpha$ 1,3-difucosylated disialo structure was observed, supporting the view of the preferential action of FT4 on nonsialylated Gal( $\beta$ 1 $\rightarrow$ 4)GlcNAc-R structures.

**FT5 cells** secrete  $\beta$ -TP with oligosaccharides modified preponderantly with the sLex motif, but also Lex-containing structures are formed. They were detected as  $\alpha$ 1,3-difu-



**Figure 5.** Comparison of fucosylated oligosaccharide antennae of  $\beta$ -TP secreted from cell lines cotransfected with human fucosyltransferases III–VII (compare also Table 4)

cosylated asialo and  $\alpha$ 1,3-difucosylated monosialo oligosaccharides. In contrast to the situation found for the monosialo-monofuco oligosaccharides from FT7 and FT4 cells, the  $\alpha$ 1,3-monofucosylated monosialo oligosaccharides from FT5 cells were found to consist of a mixture of Lex and sLex containing structures.

In a previous study [34], we have reported that human **FT3** from BHK-21 cells does not fucosylate type **II**  $N$ -acetylglucosamine structures in several glycoproteins when incubated *in vitro*. Furthermore, we could clearly demonstrate that, with bovine fetuin as a substrate, only the triantennary oligosaccharide containing one type **I** branch ( $\text{Gal}(\beta 1 \rightarrow 3)\text{GlcNAc}$ ) is modified with  $\alpha$ 1,4-linked Fuc *in vitro*, although an 8-fold higher type **II** acceptor concentration ( $\text{Gal}(\beta 1 \rightarrow 4)\text{GlcNAc-R}$  branches) was present during the experiment [34]. Similarly, we could show that no sLex or Lex *in vitro* activity was present in extracts of FT3 cells when using low molecular weight type **II** oligosaccharide acceptors (see also Table 4). These findings have been further confirmed in a recent publication from our group [54] describing the failure to *in vitro* fucosylate triantennary type **II** oligosaccharides with large amounts of a purified recombinant FT3 preparation. For the *in vivo* activity of the Golgi form of the enzyme, almost no fucosylation of asialo branches was observed in  $\beta$ -TP oligosaccharides from FT3-transfected cells. Using ESI-MS/MS and methylation analysis, we confirmed that only the sLex and no sLea structure was present in the oligosaccharides of coexpressed  $\beta$ -TP. Since only 11% of the total oligosaccharide antennae of  $\beta$ -TP from FT3 cells were modified with peripheral fucose compared to up to 50% of the total anten-

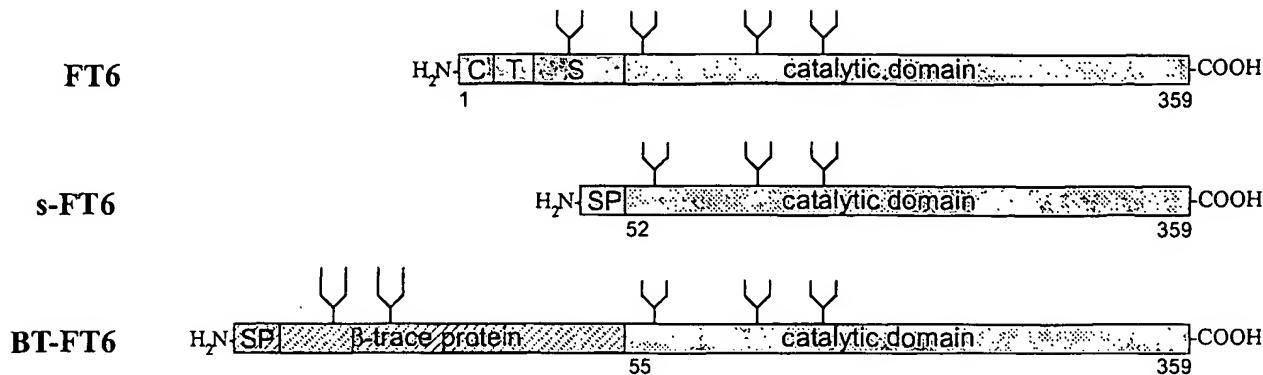
nae in  $\beta$ -TP from cells transfected with FT4-FT7 (cf. Fig. 5), we conclude that FT3 preferentially acts *in vivo* as a type **I** chain-specific transferase which is in agreement with the *in vitro* data published previously [34,54].

The results of our *in vivo* specificity studies of recombinant human  $\alpha$ 1,3/4-FTs indicate that each of the enzymes exhibits a specific fucosylation characteristics with type **II** complex  $N$ -glycan chains on coexpressed human  $\beta$ -TP as is exemplified by the different sLex/Lex ratios: FT7 (only sLex) > FT3 (14:1) > FT5 (3:1) > FT6 (1.1:1) > FT4 (1:7) [35]. Furthermore, from the results obtained, recombinant human FT6 turns out to have a high *in vivo* preference to form  $\alpha$ 1,3-difucosylated structures with all three, asialo, mono- and disialo diantennary acceptor oligosaccharides. A similar high preference for the synthesis of  $\alpha$ 1,3-difucosylated diantennary glycans is only detected for FT4 with the asialo structures (compare Table 5). Apart from its strict specificity towards  $\alpha$ 2,3-sialylated antennae, FT7 appears to have very similar preference for both,  $\alpha$ 1,3-mono- and  $\alpha$ 1,3-di-Fuc-transfer onto  $N$ -linked oligosaccharides, whereas FT3 and FT5 predominantly attach a single peripheral Fuc residue to diantennary  $N$ -glycans.

#### Human FT6 requires Golgi membrane localization for its *in vivo* activity

In a previous publication we have reported that the wild-type Golgi form of human FT3 is intracellularly cleaved in stably transfected BHK-21 cells and the catalytically active fragment can be detected by *in vitro* assays and Western blotting in the cell supernatant [34]. Similar observations have been published for  $\alpha$ 2,6-sialyltransferase [60],  $\beta$ 1,4-galactosyltransferase [61],  $\alpha$ 1,3-galactosyltransferase [62], polypeptide  $\alpha$ 1 $\rightarrow$ O GalNAc-transferase [63],  $G_{M2}$  synthase ( $\beta$ 1,4-GalNAc-transferase) [64] and FT6 [65]. The enzymes responsible for this proteolytical cleavage have been proposed to be cathepsin-like proteases or serine proteases, respectively. We have found that recombinant human FT6 is secreted by two different BHK-21 cell lines and from CHO DHFR cells [35], as shown in Table 4. While FT7 and FT5 were found to be resistant to proteolysis in transfected BHK-21 cells, we have also observed secreted forms of FT4 in supernatants of cells transfected with wild-type human FT4. It is important to consider the proteolytically cleaved enzyme forms when describing the *in vivo* specificity of glycosyltransferases, since it is known that also from natural cells/tissues considerable amounts of soluble forms have been detected in the medium of cells or in body fluids in certain diseases [66,67].

We have addressed the question of a possible contribution to the *in vivo* activity of soluble forms by engineering of cells that express variants of human FT6 lacking the cytoplasmic and transmembrane domain and part of the stem region (s-FT6), as depicted in Figure 6. In addition, we have constructed a chimeric secretable protein (BT-FT6) by fu-



**Figure 6.** Schematic representation of the protein domain structures of wild-type human FT6 and soluble variants. C, T, S denote the cytoplasmic, transmembrane and stem regions; SP = signal peptide; the number of potential *N*-glycosylation sites are indicated by symbols

sion of the full-length human  $\beta$ -TP sequence to the N-terminus of the catalytic domain of human FT6 which resulted in a 70-fold overexpression of catalytic activity. Our results obtained with the coexpression of s-FT6 together with  $\beta$ -TP indicate that enzymes secreted along the secretory pathway can be considered to not contribute to the *in vivo* functional activity of the enzyme, since only after about 20-fold overexpression of s-FT6 (cell line s-FT6(II), compare Table 4), we were able to detect very small amounts of fucosylation of  $\beta$ -TP, and even 70-fold overexpressed BT-FT6 results in a low  $\alpha$ 1,3-fucosylation of  $\beta$ -TP, as shown in Table 5. Cho and Cummings [68] found by lectin binding that a recombinant, soluble  $\alpha$ 1,3-galactosyltransferase (lacking the transmembrane and cytoplasmic domain) is functionally active *in vivo* when expressed at slightly higher levels than the full-length form of the enzyme. The reason for this discrepancy is unknown; however, that truncated forms of glycosyltransferases in general do not contribute significantly to the *in vivo* specificity of the enzyme towards secreted glycoproteins is supported by our finding that a recombinant soluble form of human ST6Gal I does not modify co-secreted  $\beta$ -TP in BHK-21 cells. This finding is confirmed by a recent publication [69] where the authors describe that soluble forms of recombinant  $\beta$ 1,4-GalNAc-transferase or ST6Gal I were not at all or significantly less efficient *in vivo* than their membrane-bound counterparts. It should be emphasized that in natural tissues or cells transferase expression levels are much lower than those that can be achieved by transfection of cells with corresponding plasmids when the gene is under the control of a strong promoter.

Interestingly, in addition to the very low fucosylation efficiency of the high enzyme activity expressing s-FT6(II) cell line in our studies, also the fucosylation pattern of  $\beta$ -TP oligosaccharides was different with a higher proportion of  $\alpha$ 1,3-monofucosylated structures observed over the  $\alpha$ 1,3-difucosylated oligosaccharides which are the major *N*-glycans synthesized by cells transfected with the full-length form of FT6 [35]. This then supports the view of the importance of the cytoplasmic, transmembrane and stem region (CTS-region) not only for the *in vivo* functional activity of glycosyltransferases, but also for their *in vivo* substrate specificities. In this context, it seems attractive to speculate that the CTS-region is also involved in the addressing of glycosyltransferases into different subcompartments of the biosynthetic glycosylation pathway of cells. The CTS polypeptide domains would be responsible for the targeting of the recombinantly expressed FT6 and FT4 to subcompartments where they can compete with the BHK cell endogenous ST3Gal III/IV for the same acceptor substrate since the sialylation state is lower for the *N*-glycans of  $\beta$ -TP secreted from the transfected cells (and as mentioned above, this indicates that ST3Gal III and ST3Gal IV do not recognize Lex motifs *in vivo*). The targeting properties of the FT6 CTS-region should result in an intracellular broader distribution of this enzyme functional activity and its overlapping with ST3Gal III/IV. FT4 should be targeted into an earlier Golgi subcompartment before the  $\alpha$ 2,3-STs modify the acceptor Gal( $\beta$ 1 $\rightarrow$ 4)GlcNAc-R substrate. Likewise, the CTS-region should direct FT7 and FT5 into a later functional compartment than the BHK cell endogenous

$\alpha$ 2,3-STs which must provide the properly sialylated oligosaccharide precursor substrates.

#### Engineering of cells by stable transfection with human Gal( $\beta$ 1 $\rightarrow$ 4)GlcNAc-R $\alpha$ 2,6-sialyltransferase

BHK-21 cells and CHO cells do not express *N*-glycan-specific  $\alpha$ 2,6-sialyltransferases (see Table 1). Therefore, glycoproteins with this typical human serum-type sialylation characteristic are not synthesized by these host cell lines. The stable transfection of cells with plasmids encoding human ST6Gal I [70] seems to be an attractive way to manipulate host cell lines for the production of this human serum-type carbohydrate structural motif. This experimental approach is justified by considering data published for the *in vitro* specificity of ST6Gal I as well as structural studies on natural glycoproteins. However, it has to be considered that the newly introduced enzyme competes with the host cell endogenous  $\alpha$ 2,3-STs (ST3Gal III/IV) for the same precursor substrate Gal( $\beta$ 1 $\rightarrow$ 4)GlcNAc-R as has been discussed above for human FT6 and FT4. In order to evaluate such a competition, we have stably transfected BHK-21B cells with a plasmid encoding the Golgi-resident form of human ST6Gal I and have coexpressed human  $\beta$ -TP as a secretory model glycoprotein.

As depicted in Figure 3, human  $\beta$ -TP from wild-type BHK cells contains simple diantennary chains which are  $\alpha$ 2,3-disialylated, monosialylated, or asialo in a ratio of 70:25:5 [16,35]. BHK-21B cells were stably transfected with plasmids encoding human  $\beta$ -TP and human ST6Gal I (*cf.* Fig. 2) and were used for the subsequent production of  $\beta$ -TP. Isolation of the secreted  $\beta$ -TP from cell supernatants was performed by a single step using immunoaffinity chromatography with a polypeptide-specific monoclonal antibody raised against  $\beta$ -TP [55]. Careful structural analysis of the *N*-linked oligosaccharides of the recombinant  $\beta$ -TP

by complementary techniques (MALDI/TOF-MS, HPAE-PAD and NMR methods [16]) was performed and we found that monosialylated and disialylated glycans were present in a ratio of 1:5. As shown in Figure 7, 60% of the disialylated oligosaccharides contained both,  $\alpha$ 2,3- and  $\alpha$ 2,6-linked NeuAc. The  $\alpha$ 2,6-linked NeuAc was preferentially attached to the Gal( $\beta$ 1 $\rightarrow$ 4)GlcNAc( $\beta$ 1 $\rightarrow$ 2)Man ( $\alpha$ 1 $\rightarrow$ 3) branch. This indicates that the newly introduced ST6Gal I competes with the endogenous ST3Gal III/IV. Since the ST6Gal I from bovine colostrum has been reported to act preferentially on the Man-3 branch of diantennary glycans [71], we could confirm the specificity of the human enzyme by our *in vivo* experiments. Similarly, we showed successful  $\alpha$ 2,6-sialylation of di- and triantennary oligosaccharides of recombinant human AT III secreted by BHK-21B cells coexpressing ST6Gal I (a ratio of  $\alpha$ 2,3- to  $\alpha$ 2,6-linked NeuAc of 1:2 was determined here by integration of the NMR signals of the axial and equatorial H-3 protons of NeuAc in the total oligosaccharide mixture [16]).

#### Human ST6Gal I transfers NeuAc in $\alpha$ 2,6-linkage to GalNAc( $\beta$ 1 $\rightarrow$ 4)GlcNAc-R motifs *in vivo*

We have previously identified a BHK-21A cell clone which synthesizes large amounts of GalNAc( $\beta$ 1 $\rightarrow$ 4)GlcNAc-R motifs in addition to the common type II Gal( $\beta$ 1 $\rightarrow$ 4)GlcNAc-R structures on secretory glycoproteins [16,17]. Recombinant glycoproteins secreted from this host cell line are found to be undersialylated because the endogenous ST3Gal III/IV do not recognize terminal GalNAc( $\beta$ 1 $\rightarrow$ 4)GlcNAc residues as a substrate. This offered the possibility to investigate if the transfection of this cell line with human ST6Gal I would result in a higher degree of sialylation of recombinant EPO, since from *in vitro* experiments it has been postulated that the enzyme also recognizes LacdiNAc

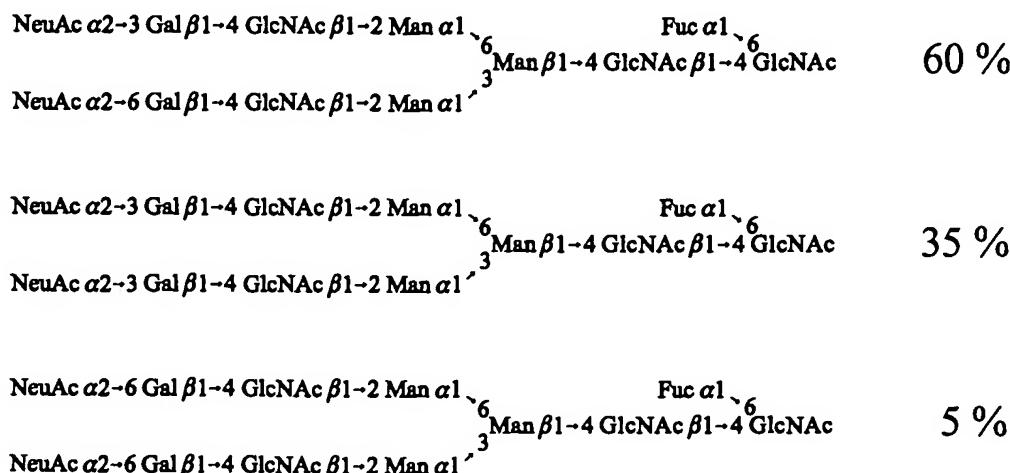


Figure 7. Disialylated *N*-linked oligosaccharide chains of recombinant  $\beta$ -TP expressed from ST6Gal I-transfected BHK-21B cells contain large amounts of  $\alpha$ 2,6-linked NeuAc.

motifs [72,73]. Human EPO expressed from wild-type BHK-21A cells contains large amounts of the unsialylated diantennary oligosaccharides with two terminal GalNAc( $\beta$ 1 $\rightarrow$ 4)GlcNAc motifs as well as tri- and tetrantennary chains with 1,2 or 3 GalNAc substitutions [17]. When cells are cotransfected with ST6Gal I (15-fold higher expression based on mRNA level and 5-fold higher enzyme activity with Gal( $\beta$ 1 $\rightarrow$ 4)GlcNAc-O-(CH<sub>2</sub>)<sub>8</sub>COOCH<sub>3</sub> as an acceptor over the endogenous  $\alpha$ 2,3-STs), we found asialo,  $\alpha$ 2,6-mono and  $\alpha$ 2,6-disialylated derivatives of the biantennary di-GalNAc oligosaccharides in a ratio of 0.1:1:0.2 based on the MALDI/TOF signals obtained from the reduced and permethylated native glycan pool of the purified EPO product (signals for [M + K<sup>+</sup>] at m/z 2360, 2721 and 3083, respectively) and methylation data [17]. This indicates that the GalNAc( $\beta$ 1 $\rightarrow$ 4)GlcNAc-R branches are efficiently recognized as substrates for ST6Gal I *in vivo*. The cell line therefore allows for the production of recombinant glycoproteins with a *human-type* NeuAc( $\alpha$ 2 $\rightarrow$ 6)GalNAc( $\beta$ 1 $\rightarrow$ 4)GlcNAc-R structural motif (see Fig. 8) that is frequently found in glycoproteins secreted from human kidney tissue [74–76].

Taken together, the expression of recombinant human  $\alpha$ 2,6-sialyltransferase increases the sialylation state of glycoproteins secreted from BHK-21A and BHK-21B cells as we have shown for human  $\beta$ -TP, AT III and EPO [16,17]. However, the human ST6Gal I has its limitations since the enzyme has a high preference for the Man-3 branch of oligosaccharides and, due to the competition with the endogenous  $\alpha$ 2,3-STs, the final products contain mixtures of  $\alpha$ 2,3/6-sialylated oligosaccharides. Also many natural human glycoproteins have both NeuAc linkages, and at present it is unknown if therapeutic glycoproteins that are exclusively modified with  $\alpha$ 2,6-linked NeuAc would be advantageous over those with a mixture of  $\alpha$ 2,3/6-linked NeuAc.

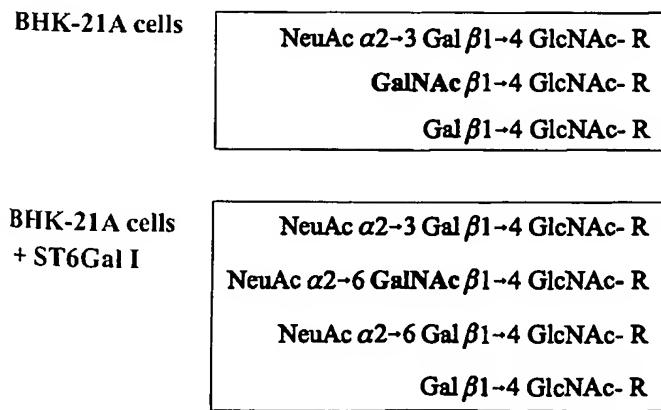


Figure 8. Terminal structural motifs in oligosaccharides of recombinant EPO from wild-type BHK-21A cells and human ST6Gal I-transfected BHK-21A cells

## Future perspectives

By using recombinant DNA technology, we are now able to efficiently manipulate the glycosylation capacity of cells to be used as new stable cell factories for biotechnological processes. However, we need to understand in much more detail basic regulatory phenomena underlying the complex interaction of the intracellular enzyme machinery that is involved in the biosynthesis of glycoconjugates. All theories and current models of compartmentalization of the cellular glycosylation pathways rely on experimental data obtained by immuno-localization of the enzymes [77–79]. In our opinion, more detailed knowledge about the *in vivo* functional localization of the glycosylation machinery of cells is required and this has to be approached experimentally.

The importance of the CTS-region of glycosyltransferases not only for their *in vivo* function, but also for their *in vivo* specificity has been addressed for the human  $\alpha$ 1,3/4-fucosyltransferases in this review. In this context, it is noteworthy that polysialyltransferase (ST8Sia IV) from humans or CHO cells possesses a very short transmembrane domain of only 13 amino acid residues [80,81] which apparently does not fit to the lipid bilayer thickness model proposed for the intracellular targeting of transmembrane proteins [78,82]. Nevertheless, according to the concept of a sequential action of glycosyltransferases, ST8Sia IV should be localized within a late Golgi compartment where it can get access to its  $\alpha$ 2,3-sialylated precursor substrate, and therefore, the CTS-region of this transferase attached to the catalytic domain of, e.g., FT6 should direct this enzyme into a location where it should synthesize higher amounts of sLex with coexpressed  $\beta$ -TP. Our results, however, obtained for this ST8Sia IV-FT6 fusion protein coexpressed together with human  $\beta$ -TP in BHK-21 cells, showed an oligosaccharide pattern for the reporter glycoprotein that was almost indistinguishable from that obtained from cells cotransfected with wild-type FT6 [83].

Other important considerations include:

- identification of *in vivo* functional cellular subcompartments at the molecular level of transferases and nucleotide sugar transporters
- intracellular turnover and the posttranslational modification of the enzymes
- dynamics of acceptor substrate and transferase transport within Golgi subcompartments
- role of splicing variants of glycosyltransferase/glycosidase genes

This is of general significance in all cases where recombinant glycobiology is used not only for the construction of “improved” cell factories for the production of more efficient and safer glycotherapeutics or recombinant retroviral vectors for gene therapy with oligosaccharide-based addressing signals, but also when it is intended to modify by

genetic/metabolic engineering the surface of *ex vivo* propagated human primary cells destined for *in vivo* therapy in clinics.

### Acknowledgments

The work described was partially supported by the EU-grant BIO2-CT94-3069 and a DAAD-grant to H.S.C.

### References

- 1 Varki A (1993) *Glycobiology* 3: 97–130.
- 2 Berman PW, Lasky LA (1985) *Trends Biochem Tech* 3: 51–3.
- 3 Kobata A (1992) *Eur J Biochem* 209: 483–501.
- 4 Schachter H (1994) In *Molecular Glycobiology* (Fukuda M, Hindsgaul O, eds.), pp 88–162. Oxford, UK: Oxford University Press.
- 5 Hoffmann A, Nimtz M, Wurster U, Conradt HS (1994) *J Neurochem* 63: 2185–196.
- 6 Hoffmann A, Nimtz M, Getzlaff R, Conradt HS (1995) *FEBS Lett* 359: 164–68.
- 7 Pohl S, Hoffmann A, Rüdiger A, Nimtz M, Jaeken J, Conradt HS (1997) *Glycobiology* 7: 1077–84.
- 8 Conradt HS, Lindenmaier W, Ausmeier M, Hauser H, Dittmar KEJ (1986) *Carbohydr Res* 49: 443–50.
- 9 Conradt HS, Egge H, Peter-Katalinic J, Reiser W, Siklosi T, Schaper K (1987) *J Biol Chem* 262: 14600–605.
- 10 Mutsaers JHGM, Kamerling JP, Devos R, Guisez Y, Fiers W, Vliegenthart JFG (1986) *Eur J Biochem* 156: 651–54.
- 11 Conradt HS, Hofer B, Hauser H (1990) *Trends Glycosci Glycotechnol* 2: 168–81.
- 12 Goochee CF, Monica T (1990) *Bio/Technology* 8: 421–27.
- 13 Goochee CF, Gramer MJ, Andersen DC, Bahr JB, Rasmussen JR (1991) *Bio/Technology* 9: 1347–55.
- 14 Jenkins N, Parekh RB, James DC (1996) *Nature Biotechnol* 14: 975–81.
- 15 Grabenhorst E, Nimtz M, Conradt HS, unpublished results.
- 16 Grabenhorst E, Hoffmann A, Nimtz M, Zettlmeissl G, Conradt HS (1995) *Eur J Biochem* 232: 718–25.
- 17 Schlenke P, Grabenhorst E, Nimtz M, Conradt HS (1998) *Cytotechnology*, in press.
- 18 Grabenhorst E, Hofer B, Nimtz M, Jäger V, Conradt HS (1993) *Eur J Biochem* 215: 189–97.
- 19 Ackermann M (1995) Doctoral thesis, Technical University of Braunschweig, Germany.
- 20 Ackermann M, Nimtz M, Grabenhorst E, Conradt HS, Jäger V (1995) In *The Baculovirus and Insect Cell Gene Expression Conference*, Pinehurst, NC, USA (abstract).
- 21 Knepper TP, Arbogast B, Schreurs J, Deinzer ML (1992) *Biochemistry* 31: 11651–59.
- 22 James DC, Freedman RB, Hoare M, Ogonah OW, Rooney BC, Larionov OA, Dobrovolsky VN, Lagutin OV, Jenkins N (1995) *Bio/Technology* 13: 592–96.
- 23 Grabenhorst E (1994) Doctoral thesis, Technical University of Braunschweig, Germany.
- 24 Dittmar KEJ, Conradt HS, Hauser H, Hofer B, Lindenmaier W (1989) In *Advances in Protein Design* (Blöcker H, Collins J, Schmidt RD, Schomburg D, eds.), GBF-Monographs vol. 12, pp 145–56. Weinheim, New York: VCH Publishers.
- 25 Kagawa Y, Takasaki S, Utsumi J, Hosoi K, Shimizu H, Kochibe N, Kobata A (1988) *J Biol Chem* 263: 17508–15.
- 26 Zettlmeissl G, Conradt HS, Nimtz M, Karges P (1989) *J Biol Chem* 264: 21153–59.
- 27 Sasaki H, Bothner B, Dell A, Fukuda M (1987) *J Biol Chem* 262: 12059–76.
- 28 Takeuchi M, Takasaki S, Miyazaka H, Kato T, Hoshi S, Kochibe N, Kobata A (1988) *J Biol Chem* 263: 3657–63.
- 29 Nimtz M, Wray V, Augustin M, Klöppel K-D, Conradt HS (1993) *Eur J Biochem* 213: 39–56.
- 30 Hokke CH, Bergwerff AA, van Dedem GWK, Kamerling JP, Vliegenthart JFG (1995) *Eur J Biochem* 228: 981–1008.
- 31 Grabenhorst E (1991) Diploma thesis, Technical University of Braunschweig, Germany.
- 32 Conradt HS, Gawlitzeck M, Grabenhorst E, Hoffmann A, Nimtz M, Oltmanns-Bleck F, Pohl S (1997) In *Medicinal Chemistry: Today and Tomorrow* (Yamazaki M, ed) pp 205–12. Blackwell Science.
- 33 Conradt HS, Nimtz M, Dittmar KEJ, Lindenmaier W, Hoppe J, Hauser H (1989) *J Biol Chem* 264: 17368–73.
- 34 Costa J, Grabenhorst E, Nimtz M, Conradt HS (1997) *J Biol Chem* 272: 11613–21.
- 35 Grabenhorst E, Nimtz M, Costa J, Conradt HS (1998) *J Biol Chem*, in press.
- 36 Kukowska-Latallo JF, Larsen RD, Nair RP, Lowe JB (1990) *Genes Dev* 4: 1288–1303.
- 37 Goelz S, Hession C, Goff D, Griffiths B, Tizard R, Newman B, Chi-Rosso G, Lobb R (1990) *Cell* 63: 1349–56.
- 38 Weston BW, Nair RP, Larsen RD, Lowe JB (1992) *J Biol Chem* 267: 4152–60.
- 39 Koszdin KL, Bowen BR (1992) *Biochem Biophys Res Commun* 187: 152–57.
- 40 Weston BW, Smith PL, Kelly RJ, Lowe JB (1992) *J Biol Chem* 267: 24575–84.
- 41 Sasaki K, Kurata K, Funayama K, Nagata M, Watanabe E, Ohta S, Hanai N, Nishi T (1994) *J Biol Chem* 269: 14730–37.
- 42 Varki A (1994) *Proc Natl Acad Sci USA* 91: 7390–97.
- 43 Lasky LA (1995) *Ann Rev Biochem* 64: 113–39.
- 44 McEver RP, Moore KL, Cummings RD (1995) *J Biol Chem* 270: 11025–28.
- 45 Kansas GS (1996) *Blood* 88: 3259–87.
- 46 de Graaf TW, van der Stelt ME, Anbergen MG, van Dijk W (1993) *J Exp Med* 177: 657–66.
- 47 Brinkman-van der Linden EC, de Haan PF, Havenga EC, van Dijk W (1998) *Glycoconj J* 15: 177–82.
- 48 de Vries T, Srnka CA, Palcic MM, Swiedler SJ, van den Eijnden DH, Macher BA (1995) *J Biol Chem* 270: 8712–22.
- 49 de Vries T, Palcic MP, Schoenmakers PS, van den Eijnden DH, Joziasse DH (1997) *Glycobiology* 7: 921–27.
- 50 Shinoda K, Morishita Y, Sasaki K, Matsuda Y, Takahashi I, Nishi T (1997) *J Biol Chem* 272: 31992–97.
- 51 Britten CJ, van den Eijnden DH, McDowell W, Kelly VA, Witham SJ, Edbrooke MR, Bird MI, de Vries T, Smithers N (1998) *Glycobiology* 8: 321–27.
- 52 Natsuka S, Gersten KM, Zenita K, Kannagi R, Lowe JB (1994) *J Biol Chem* 269: 16789–94.
- 53 Kumar R, Potvin B, Muller WA, Stanley P (1991) *J Biol Chem* 266: 21777–83.

- 54 Nimitz M, Grabenhorst E, Gambert U, Costa J, Wray V, Morr M, Thiem J, Conradt HS (1998) *Glycoconj J* **15**: 873–83.
- 55 Hoffmann A, Nimitz M, Conradt HS (1997) *Glycobiology* **7**: 499–506.
- 56 Smith DF, Larsen RD, Mattox S, Lowe JB, Cummings RD (1990) *J Biol Chem* **265**: 6225–34.
- 57 Sueyoshi S, Tsuboi S, Sawada-Hirai R, Dang UN, Lowe JB, Fukuda M (1994) *J Biol Chem* **269**: 32342–50.
- 58 Lowe JB, Kukowska-Latallo JF, Nair RP, Larsen RD, Marks RM, Macher BA, Kelly RJ, Ernst LK (1991) *J Biol Chem* **266**: 17467–77.
- 59 Gersten K, Natsuka S, Trinchera M, Petryniak B, Kelly RJ, Hiraiwa N, Jenkins NA, Gilbert DJ, Copeland NG, Lowe JB (1995) *J Biol Chem* **270**: 25047–56.
- 60 Weinstein J, Lee EU, McEntee K, Lai P-H, Paulson JC (1987) *J Biol Chem* **262**: 17735–43.
- 61 Masri KA, Appert HE, Fukuda MN (1988) *Biochem Biophys Res Commun* **157**: 657–63.
- 62 Larsen RD, Rajan VP, Ruff MM, Kukowska-Latallo J, Cummings RD, Lowe JB (1989) *Proc Natl Acad Sci USA* **86**: 8227–31.
- 63 Homa FL, Hollander T, Lehman DJ, Thomsen DR, Elhammer AP (1993) *J Biol Chem* **268**: 12609–16.
- 64 Jaskiewicz E, Guofen Zhu, Bassi R, Darling DS, Young WW Jr (1996) *J Biol Chem* **271**: 26395–403.
- 65 Borsig L, Katopodis AG, Bowen BR, Berger EG (1998) *Glycobiology* **8**: 259–68.
- 66 Kaplan HA, Woloski BMRNJ, Hellman M, Jamieson JC (1983) *J Biol Chem* **258**: 11505–9.
- 67 Uejima T, Uemura M, Nozawa S, Narimatsu H (1992) *Cancer Res* **52**: 6158–63.
- 68 Cho SK, Cummings RD (1997) *J Biol Chem* **272**: 13622–28.
- 69 Zhu G, Allende ML, Jaskiewicz E, Qian R, Darling DS, Worth CA, Colley KJ, Young WW Jr (1998) *Glycobiology* **8**: 831–40.
- 70 Grundmann U, Nerlich C, Rein T, Zettlmeissl G (1990) *Nucl Acids Res* **18**: 667.
- 71 Joziasse DH, Schiphorst WECM, van den Eijnden DH, Van Kuik JA, Van Halbeek H, Vliegenthart JFG (1987) *J Biol Chem* **262**: 2025–33.
- 72 Nemansky M, van den Eijnden DH (1992) *Biochem J* **287**: 311–16.
- 73 Hokke CH, van der Ven JGM, Kamerling JP, Vliegenthart JFG (1993) *Glycoconj J* **10**: 82–90.
- 74 Weisshaar G, Hiyama J, Renwick AGC, Nimitz M (1991) *Eur J Biochem* **195**: 257–68.
- 75 Yan SB, Chao YB, van Halbeek H (1993) *Glycobiology* **3**: 597–608.
- 76 Bergwerff AA, Van Oostrum J, Kamerling JP, Vliegenthart JFG (1995) *Eur J Biochem* **228**: 113–26.
- 77 Colley KJ (1997) *Glycobiology* **7**: 1–13.
- 78 Munro S (1998) *Trends Cell Biol* **8**: 11–15.
- 79 Lowe M, Nakamura N, Warren G (1998) *Trends Cell Biol.* **8**: 40–44.
- 80 Nakayama J, Fukuda MN, Fredette B, Ranscht B, Fukuda M (1995) *Proc Natl Acad Sci USA* **92**: 7031–35.
- 81 Eckhardt M, Mühlhoff M, Bethe A, Koopman J, Frosch M, Gerardy-Schahn R (1995) *Nature* **373**: 715–18.
- 82 Bretscher MS, Munro S (1993) *Science* **261**: 1280–81.
- 83 Grabenhorst E, Nimitz M, Conradt HS, manuscript in preparation.

Accepted 12 February 1999.

# Glycoconjugate Journal

Volume 16 Number 2 February 1999

Official Journal of the

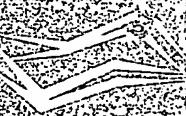
International Glycoconjugate

Organization

VOLUME 16 · NUMBER 2 · FEBRUARY 1999 · PAGES 81–182

ISSN 0282-0080

Chief Editor  
G.A. Turner



Kluwer Academic Publishers

ISSN: 0282-0080 CODEN: GLJOEW

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record.**

## **BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- BLACK BORDERS**
- IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- FADED TEXT OR DRAWING**
- BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- SKewed/SLANTED IMAGES**
- COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- GRAY SCALE DOCUMENTS**
- LINES OR MARKS ON ORIGINAL DOCUMENT**
- REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- OTHER:** \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**